

Factors affecting the viability of human platelets.

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Abstract.

Human platelets die *in vitro* in a caspase-independent manner with features of necrosis. Little is known about the factors affecting the mode of platelet death or how death is brought about. The research programme described in this thesis aimed to generate novel tools for the discrimination of live from dead platelets and to evaluate the role of autophagy, temperature and sugar metabolism in the demise of platelets *in vitro*. Two dyes, calcein and FM4-64 were found to be useful for the evaluation of platelet viability. It was found that during *in vitro* storage dead platelets formed metalloproteinase-dependent aggregates and shed CD42b. Platelets died more slowly at ambient temperature than at 37°C. Platelets did not rely on exogenous glucose for viability but hypoglycaemia sensitised them to pro-apoptotic stimuli. Inhibitors of glycogen breakdown were toxic to platelets, implicating glycogen in the maintenance of platelet viability *in vitro*. Autophagy could not be implicated in the loss of platelet viability but data suggested a role for crinophagy in the maintenance of platelet function.

Declaration.

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous application for candidature for a higher degree. All work presented in this thesis, unless acknowledged otherwise, was initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

Paul S. Hartley.

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Dedication.

With love to Kirstie and Beth.

I hope that one day there will be better ways to do these things.

Abbreviations.

2DG	2-Deoxy D-Glucose
3-MA	3 Methyladenine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cPRP	Citrated PRP
CVX	Convulxin
dcPRP	Diluted cPRP
DMSO	Dimethylsulphoxide
DTS	Dense tubular system
$\Delta\Psi_m$	Mitochondrial membrane potential
$\Delta\Psi_p$	Plasma membrane potential
EDTA	Ethyldiaminetetraacetic acid
FITC	Fluorescein isothiocyanate
FM4-64	N-(3-triethylammoniumpropyl)-4-(p-diethylaminophenyl)-hexatrienyl)pyridinium dibromide
FSC	Forward scatter
GLUT	Glucose transporter
HBSS	Hanks' buffered saline solution
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl-carbocyanine iodide
kD	Kilodalton
Lamp-2	Lysosome associated membrane protein 2
LTR	Lysotracker red
mAb	Monoclonal antibody
mCCCP	Carbonyl cyanide m-chlorophenyl-hydrazone
MDC	Monodansylcadaverine

Abbreviations

MKs	Megakaryocytes
MTG	Mitotracker green
OCS	Open canalicular system
pAb	Polyclonal antibody
PAS	Periodic acid - Schiff's Reagent
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PE	Phosphatidylethanolamine
PRP	Platelet rich plasma
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
SDS PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
SSC	Side scatter
TEM	Transmission electron microscope / micrograph
TRAP	Thrombin receptor agonist peptide
vWF	von Willebrand Factor

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1 INTRODUCTION.

1.1 Platelets - an overview.

Bizzozzero described the platelet as a haemostatic cell in 1881 (Bizzozzero, 1881). Using intravital microscopy of guinea pig mesenteric venules he observed their change from resting single cells to aggregates at sites of vascular injury. Interestingly, many of his contemporaries did not believe his contention, favouring the notion that blood clots were made only of fibrin. Ironically, the confirmation of his observations nearly fifty years later did little to influence the scientific community that platelets had anything to do with clot formation (Burk and Tait, 1926). However it has since become dogma that platelets perform the crucial role of maintaining the integrity of our blood vessels. Platelets are now arguably one of the best known of all human cell types.

Despite the enormous clinical and academic importance of platelets it is surprising that little is known about their senescence and death. Platelet senescence both *in vitro* and *in vivo* has been associated with an apoptotic-like phenotype (Brown et al., 2000; Pereira et al., 2002; Clarke, 2002; Clarke et al., 2003) and loss of functional responsiveness to collagen (Hirsh *et al.*, 1968) and thrombin (Peng *et al.*, 1994). Although it is known that apoptosis leads to a prothrombogenic phenotype due to phosphatidylserine (PS) exposure (Bombeli *et al.*, 1997) the net result of these gains and losses of thrombotic potential to haemostasis is unknown but an inability to remove senescent platelets from circulation is likely to have a complex but overall negative effect on haemostasis and platelet-mediated inflammatory responses.

In evolutionary terms platelets are relatively modern cell types. They appear to have evolved from nucleated haemocytes that are found within the haemolymph of lower organisms such as invertebrates and the Horseshoe Crab *Limulus polyphemus*. Vertebrates such as birds, fish and amphibia retain nucleated haemocytes (also known as thrombocytes) and it is only mammals that are known to produce anucleate platelets. Quite why mammals evolved anucleate, specialised cells such as platelets is unknown.

The typifying features of haemocytes include their ability to adhere, aggregate, retract, undergo viscous metamorphosis (shape change), promote coagulation, promote vessel contraction and phagocytose and degrade foreign material. Although the haemostatic functions of haemocytes have been retained by mammalian platelets other features, such as phagocytosis and anti-microbial activity, seem to have been retained only at a very rudimentary level (Levin, 2002).

Human platelets are small ($\sim 3 \times 0.5 \mu\text{m}$), numerous ($\sim 300,000 \mu\text{l}^{-1}$) anucleate sub-cellular fragments that have a lifespan in the human of approximately seven to ten days. Circulating platelets are non-adhesive and discoid in shape and similar to that of the nucleated *Limulus* haemocytes that preceded them 400 million years ago. Interaction of platelets with exposed sub-endothelial matrix evokes changes to platelet surface receptors that promote firm adhesion and a complex range of signalling cascades leading to degranulation of storage granules which amplifies platelet activation, promotes platelet-platelet interactions and enzymatic activity that culminates in the plugging of damaged vessels and the cessation of haemorrhage. In abnormal situations the inappropriate activation of platelets leads to thrombotic events, increased platelet

deposition in unsuitable areas of the vasculature. Build up of platelets leads to vessel occlusion, increasing the likelihood of ischemia, infarction, stroke and death. Conversely, dysfunctional platelets are incapable of maintaining the integrity of the vasculature and bleeding disorders ensue.

1.2 Platelet ultrastructure.

Although the smallest cell type found within mammals platelets contain a surprisingly complete array of organelles and storage granules including mitochondria, lysosomes, alpha granules (containing predominantly proteinaceous material); dense granules (containing small molecules such as ADP, ATP, serotonin, calcium and magnesium); a dense tubular system (analogous to the endoplasmic reticulum of nucleated cells); a highly invaginated plasma membrane system known as the open canalicular system (OCS) and large fields of glycogen. A microtubule coil at the platelet's widest radius maintains their discoid structure (fig. 1). Platelets contain a very well characterised cytoskeletal scaffold that undergoes rapid and massive reorganisation during activation in order to bring about shape change, filopod and pseudopod extension during spreading and aggregation (fig. 2).

1.3 Platelet production.

Platelets arise from progenitor cells called megakaryocytes (MKs). MKs differentiate from pluripotent stem cells of the myeloid lineage and under several rounds of endomitosis, developing into highly characteristic large, polyploid cells. It appears that

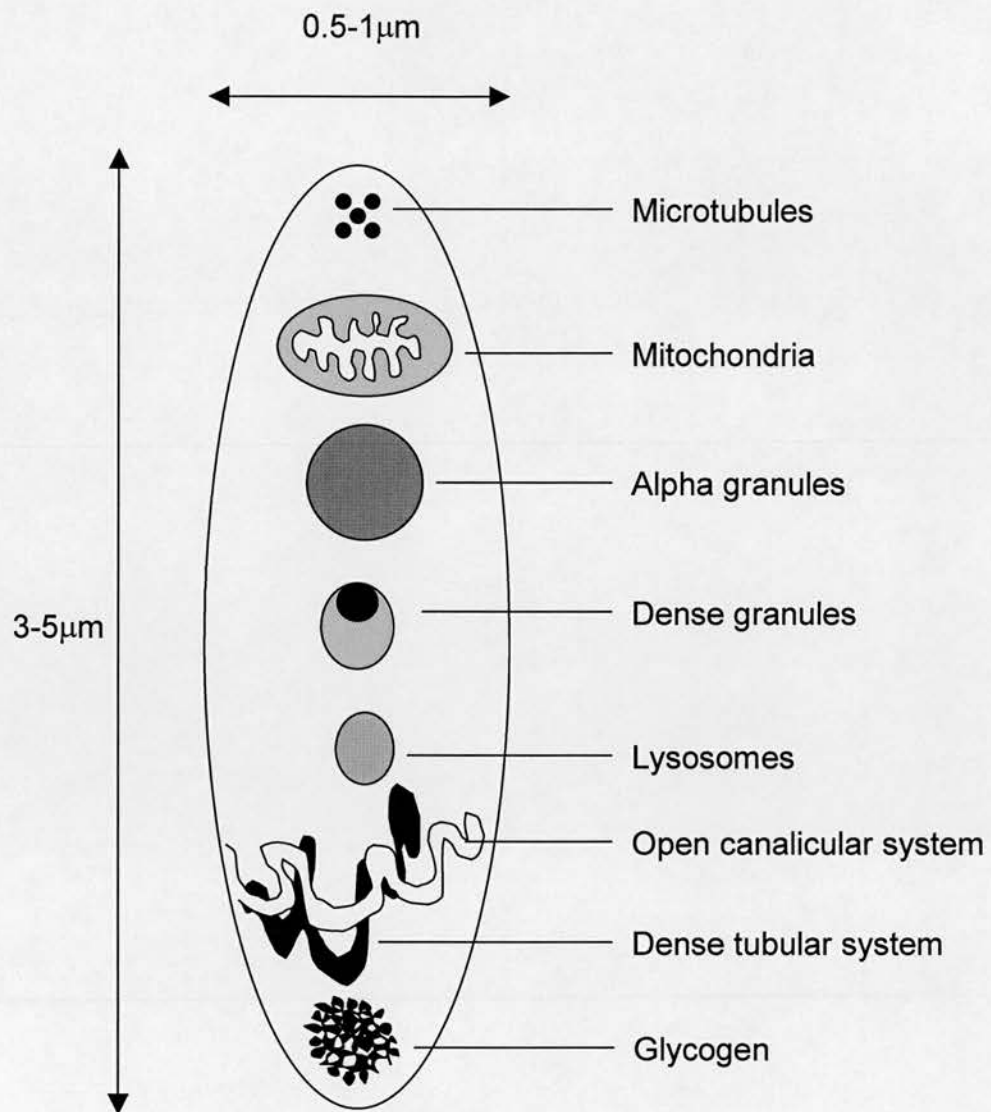


Figure 1. Schematic of platelet ultrastructure.

Although platelets are devoid of a nucleus and Golgi-ER system they contain many organelles, storage granules and cytoskeletal components. These include mitochondria, alpha and dense granules, lysosomes an open canalicular system that is in close association with the dense tubular system and glycogen.

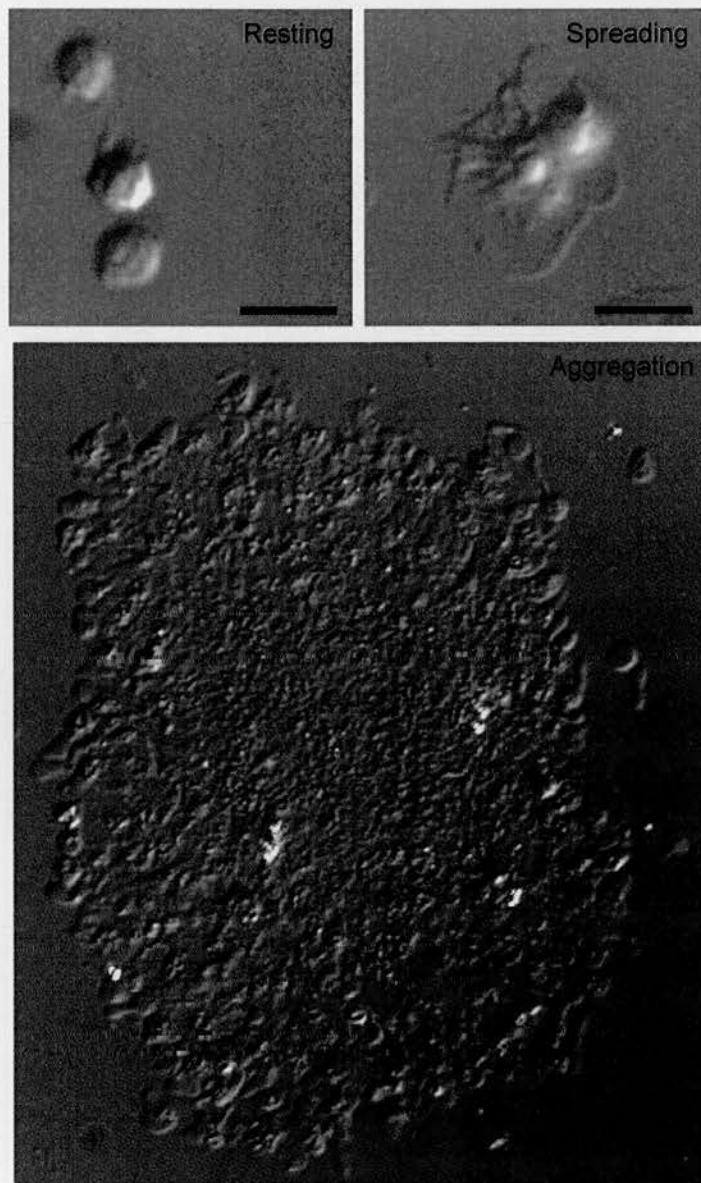


Figure 2. Micrographs illustrating gross morphology of platelet activation.

Platelets are discoid in their resting state but transform rapidly upon glass or injured vasculature. Both filopod and pseudopod formation occurs, a process known as 'spreading'. Activation of platelets in stirred suspension leads to platelet-platelet interactions and aggregate formation. Bar = 5 μ m.

the process of platelet production from MKs is not clearly understood, however development of megakaryocytes and progenitors is primarily under the control of a single growth factor, thrombopoietin (TPO) (de Sauvage *et al.*, 1996) whose cognate receptor upon the megakaryocyte (and precursors) is cMpl (Gurney *et al.*, 1994). The sites of platelet production are typically the bone marrow but it is also possible that platelets are formed in the lung and blood.

The formation of platelets from the mature megakaryocyte is an elaborate process of compartmentalised apoptosis (De Botton *et al.*, 2002; Clarke *et al.*, 2003). The cell's nuclear region and immediate cytoplasmic environment undergoes caspase-dependent programmed cell death whereas a large portion of the organelle and storage granule-containing cytoplasm is immune from the execution of this program. The remaining plasma membrane system, cytosol and organelles (including the platelet's alpha and dense granules) fragments into the subcellular particles known as platelets.

1.4 Platelet adhesion to sites of vascular injury.

Platelet functionality is based on their ability to recognise areas of damaged vasculature, remain adhered to them whilst attracting further platelets and promoting coagulation. Two receptors are of key importance in the initial tethering of platelets at high shear force to sites of vascular injury (fig. 3). Both link platelets to the matrix but do so by slightly different means. GPVI is able to bind directly to exposed fibrillar collagen and initiates the activation of further platelet receptors (most importantly the $\alpha_{IIb}\beta_3$ integrin complex) that enables firm adhesion. In contrast, GPIb-IX-V is able to bind vWF that is

bound to collagen. Both receptors therefore mediate the binding of platelets to collagen, one directly and one indirectly.

1.5 Glycoprotein Ib-IX-V.

The GPIb-IX-V complex is a heptameric complex consisting of four different proteins; glycoprotein Ib α ; glycoprotein Ib β ; glycoprotein IX and glycoprotein V; (CD42a, b, c and d, respectively) in the ratio 2:2:2:1 (fig. 4). The complex exists as linear arrays on the surface of resting platelets but as clusters on the surface of activated platelets (Hoffmeister *et al.*, 2003). The complex mediates the platelet's adhesion to damaged vessel walls at high shear force (Cranmer *et al.*, 1999). The ligand most important for high shear force interactions of platelets with vessel wall is von-Willebrand factor (vWF), a multimeric protein integral to sub-endothelial matrix but which is found also in plasma due to its secretion from activated platelets and Weibel-Palade bodies of endothelial cells. The adhesion of platelets to vWF via the GPIb-IX-V complex initiates platelet activation due to signalling events that lead to cytoskeletal rearrangements; integrin activation and calcium mobilisation. Essentially, the receptor's interaction with vWF represents a platelet 'braking system' that allows the platelet to exit from the rapid flow of blood at sites of injury. Without this interaction few other platelet-vessel wall interactions can occur, thus people that have defects in either vWF or the GPIb-V-IX receptor have a bleeding disorder.

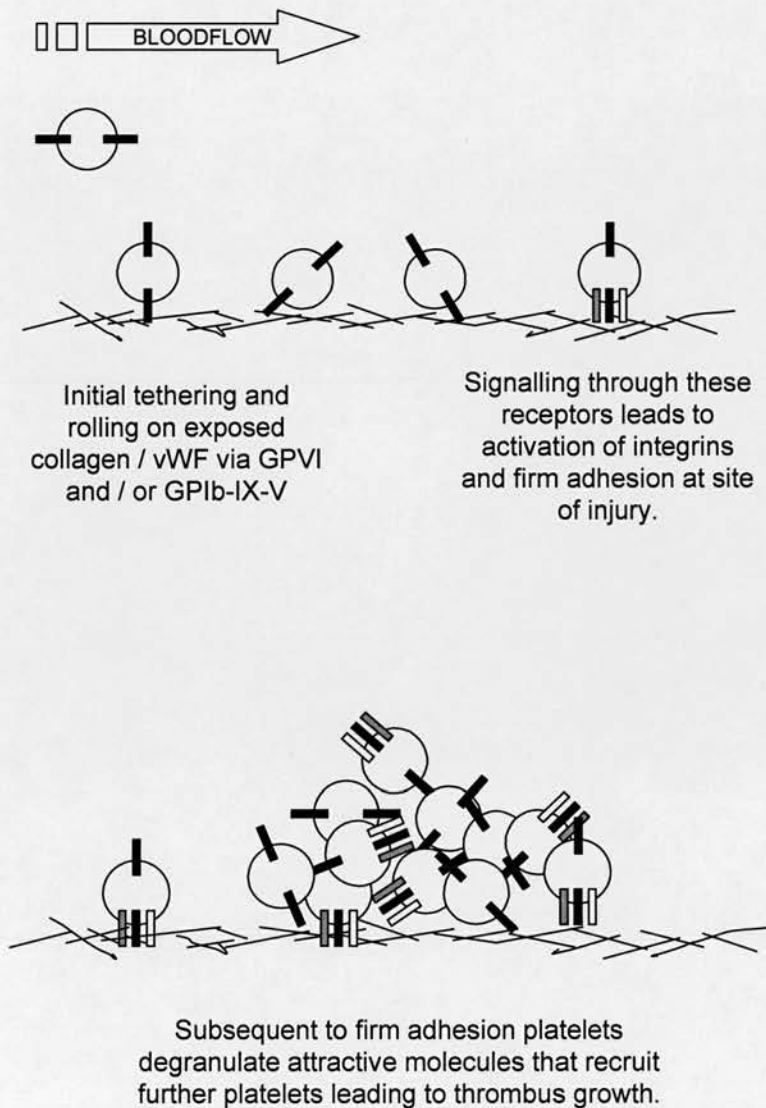


Figure 3. Platelet recruitment to sites of vascular injury.

Exposure of matrix proteins such as collagen and vWF allows resting platelets to tether and roll via GPVI and GPIb-IX-V receptors. Signalling events initiated by this interaction lead to integrin activation, further platelet-matrix interactions and firm adhesion at the site of injury. Platelet activation proceeds with degranulation of attractive molecules that recruit platelets and promotes thrombus growth.

Thrombin is known also to bind the complex, an event that, although not activating platelets directly, facilitates the proteolysis of the PAR-1 thrombin receptor (De Candia *et al.*, 2001).

Regulation of the GPIb-V-IX complex and its signalling capacity is mediated by at least four mechanisms. Adhesion to vWF and thrombin is regulated by sulfation of tyrosine residues within the ligand-binding domain. Secondly, GPIb α is heavily glycosylated beyond amino acid residue 282 in an area known as the sialomucin core. This glycosylation appears to be important for the extension of the ligand-binding domain outwards from the platelet's surface. Thirdly, although controversial, the complex is thought to reside within localised areas of the platelet plasma membrane known as rafts (Gousset *et al.*, 2002; Shrimpton *et al.*, 2002). This localisation is probably mediated by the palmitoylation of GPI β and GPIX close to their membrane spanning domains that effectively anchors them within the rafts. Lastly, phosphorylation of serine 166 within the cytoplasmic tail of GPI β by cAMP-dependent kinase PKA appears to negatively regulate cytoskeletal reorganisation (Bodnar *et al.*, 2002). Phosphorylation of serine 609 within the cytoplasmic tail of GPIb α (by an unknown kinase) appears to mediate the formation of a signalling complex containing 14-3-3 ξ that is essential for activation of the $\alpha_{IIb}\beta_3$ integrin complex (Andrews *et al.*, 2003).

Changes to the external aspect of the GPIb-IX-V complex can be evoked by cold storage (Hoffmeister *et al.*, 2003) and appear to involve both the clustering of the complex and exposure of beta-N-acetylglucosamine residues of N-linked glycans on

CD42b; this leads to the recognition and subsequent phagocytosis by liver macrophages of cold-stored platelets (Hoffmeister et al., 2003; Hoffmeister et al., 2003). The loss of CD42b from the platelet surface is known to occur during activation (Michelson *et al.*, 1991), extended *in vitro* storage (Michelson *et al.*, 1988), in response to plasmin (Michelson and Barnard, 1990) and deliberate collapse of platelets' $\Delta\Psi_m$ using protonophore (Bergmeier et al., 2003). Changes to CD42b appear therefore to be associated with a broad range of scenarios both *in vivo* and *in vitro* but as yet there is no physiological role appointed to these changes other than the speculation it may negatively regulate the binding of platelets to vWF and collagen (Furman *et al.*, 2000) or that it serves as a negative regulator of thrombin activity due to the liberation of glyocalicin, the 130kDa proteolytic fragment abundant in plasma (Jamieson *et al.*, 1980).

The initial tethering of platelets to sites of vascular injury is followed by inside-out signalling of the integrin $\alpha_{IIb}\beta_3$ and $\alpha_2\beta_1$ integrin complexes. Ligands for these receptors include the RGD-containing proteins fibrinogen, fibronectin and vitronectin but also vWF in the case of $\alpha_{IIb}\beta_3$.

1.6 The $\alpha_{IIb}\beta_3$ integrin complex.

The α_{IIb} and β_3 integrins form a 1:1 Ca^{2+} -dependent complex on platelets that constitutes the receptor for RGD-containing proteins such as fibrinogen and fibronectin (Brass *et al.*, 1985). The complex is abundantly expressed (50,000 – 80,000 copies) upon the platelet surface and is the most abundant receptor they possess. An

intracellular pool of the complex exists within the α -granules which can be surface expressed during degranulation. Platelet deposition on exposed collagen leads to signalling cascades originating from the GPIb-IX-V and GPVI receptor complexes that converge on the $\alpha_{IIb}\beta_3$ and direct a conformational change that switch it from a low to a high affinity state, facilitating the firm adhesion of platelets to the site of injury (Peerschke *et al.*, 1980). The occupancy of the integrin receptor by ligand induces outside-in signalling that further promotes intraplatelet changes such as mobilisation of calcium, cytoskeletal reorganisation and degranulation.

Mutations within the receptor or loss of the complex lead to a bleeding disorder termed Glanzmann's Thrombasthenia. Most sufferers have loss of function mutations that lead to the disorder however, a gain of function mutation that locks the receptor complex in the high affinity state has also been described (Fullard *et al.*, 2001).

An important note to make regarding this integrin complex is that it is a major target of the anticoagulant activity of EDTA. Platelets exposed to EDTA lose the ability to aggregate due to dissociation of the integrin complex (Grant and Zucker, 1978; Zucker and Grant, 1978). Removal of EDTA and the subsequent replacement of calcium fail to restore platelet aggregability unless the α -granule store of the receptor is expressed on the platelet surface (Zucker and Grant, 1978). The effect of EDTA on platelets can be observed within 10 minutes and is known to be temperature and pH dependent because dissociation does not occur within this time if platelets are incubated below 37°C and <pH 7 (Zucker and Grant, 1978). Ultrastructural changes associated with the loss of the integrin complex lead to the formation a 'zipped-up' canalicular system (so called

‘Birbeck-like granules’), a phenotype that is thought to result from homotypic interactions between α_{IIb} (Gachet et al., 1992; Gachet et al., 1993). An extracellular calcium concentration of above 10^{-6}M is sufficient for maintenance of the complex (Fujimura and Phillips, 1983; Fitzgerald and Phillips, 1985).

1.7 Phosphatidylserine (PS) exposure.

PS exposure is an important response of platelets and erythrocytes to stimuli originating at sites of vascular injury (Bever *et al.*, 1982). Increased exposure of PS allows the binding to the cell surface of activated factor V (factor Va) that leads to prothrombinase activity of factor Xa and the subsequent cleavage by thrombin of fibrinogen to form fibrin.

In resting cells the maintenance of aminophospholipids such as PS and PE on the endofacial side of the plasma membrane is mediated by ATP-dependent aminophospholipid translocase activity which can be inhibited by increased intracellular calcium concentrations, growth factor starvation and the tyrosine kinase inhibitor, $\text{VO}_{(4)}^{3-}$ (Julien *et al.*, 1995). It is thought that transbilayer movement of PS and PE to the exofacial side of the membrane is mediated by inhibition of translocase activity and a concomitant initiation of ‘scramblase’ activity. The gene encoding a human phospholipid scramblase has been recently cloned (Zhou *et al.*, 1997), however mice deficient in the PLSCR-1 gene can still scramble PS, suggesting that other ‘scrambling’ genes are available for this process (Zhou *et al.*, 2002).

Exposed PS can be detected by a number of biochemical assays including the measurement of prothrombinase activity and the binding in the presence of calcium of annexin V (Thiagarajan and Tait, 1990). The procoagulant response induced by collagen (as a soluble monomer or as an immobilised 'fibrillar' form) is superior to that caused by thrombin, ADP or immobilised fibrinogen, however there is clear synergy between agonists (Thiagarajan and Tait, 1990; Heemskerk et al., 1997). It is interesting to note that the gross morphology of collagen-activated platelets is dramatically different to that of ADP or thrombin treated platelets. The typical response of platelets to ADP or thrombin is shrinkage, granule coalescence and pseudopod formation whereas platelet spherizing, microparticle formation and PS exposure typify that induced by collagen.

1.8 Degranulation.

After the initial tethering and development of firm adhesion platelets release the contents of their storage granules. This process of exocytosis serves to amplify platelet activation at the site of injury but also recruits resting platelets from circulation. It is an elaborate process of cytoskeletal rearrangements and membrane fusion events that is far from being fully understood (Reed *et al.*, 2000). Platelets contain three major granules; the alpha and dense granules and lysosomes. Alpha granules contain proteinaceous material such as the fibrinogen, Factors V and XIII, von Willebrand Factor, thrombospondin, IgG, albumin, alpha 2-antiplasmin and alpha 2-macroglobulin. Furthermore the α -granule membrane expresses $\alpha_{IIb}\beta_3$ integrin complex and P-Selectin (Harrison and Cramer, 1993). Dense granules contain small molecules such as ATP,

GTP, ADP, GDP, phosphate, serotonin, calcium and magnesium (Reed, 2002). Lysosomes are more enigmatic granules and although containing many characteristic acid hydrolases, their contribution to platelet activation and haemostasis is not understood. The release of granule contents coincides with surface expression of the integral membrane proteins, thus degranulation of alpha and dense granules and lysosomes can be monitored by the appearance upon the platelet surface of P-selectin, CD63 or Lamp2, respectively.

1.9 Changes associated with platelet senescence.

In vivo studies of platelet senescence often rely upon manipulation of platelet populations so that 'older' populations are isolated from ever-dwindling populations of circulating platelets (ablated megakaryopoietic models). This method of analysis forces those platelets that remain in circulation to maintain the vasculature and possibly driving the few remaining platelets into a state of perpetual activity that is abnormal under physiological conditions. In contrast to this are models that rely on the clearance of all platelets with subsequent analysis of 'young' platelet populations in comparison to the normal population. Massive loss of platelets caused either by controlled bleeds or immune clearance is likely to abnormally affect platelet production, although authors using these techniques are aware of such artefacts it is difficult to interpret data in terms of *real* changes due to a platelet's time in circulation. Many authors have assumed that platelet heterogeneity is linked to their age, thus many have reported correlations between platelet size, volume, density, function and biochemical character. There is no doubt that these correlations exist but whether they are truly a reflection of a platelet's

age is uncertain. It has also been argued that platelets are not manufactured to a template and that any heterogeneity within a platelet population is likely to have arisen at the point of production. A review of some key papers follows.

One of the earliest demonstrations that platelet senescence is associated with dysfunction was documented nearly forty years ago by the injection of rabbits with $^{35}\text{SO}_4$ and collection of platelets 54 hours and 96 hours after administration of the isotope (Hirsh *et al.*, 1968). The isolated platelets were then aggregated and it was found that the older platelets adhered less well to collagen than younger platelets. In an independent clinical study of a patient lacking a plasma factor important for the production of platelets (possibly thrombopoietin), Johnson *et al* collected and analysed platelets during a 30-week period of repeated normal plasma transfusions. Platelet production was monitored after the transfusion of normal plasma, with numbers rising from $<10000/\mu\text{l}$ to $200000 - 300000/\mu\text{l}$ by day 4, peaked at $>500000/\mu\text{l}$ by day 9 and gradually fell back to baseline figures by day 21 to 23. This three-week production period corresponds to one round of megakaryocytopoiesis and thus platelets present on day 4 were younger than those present towards the end of the treatment day 21. Aggregation studies revealed that younger platelets responded better than old to low concentrations of ADP ($2\mu\text{M}$) but no difference was seen in response to supra-maximal doses of ADP ($20\mu\text{M}$) or collagen ($500\mu\text{g/ml}$), however platelet adhesion to glass was significantly impaired in the old population. Clot retraction, ultrastructure and prothrombin time were similar between young and old populations. Although this data came from a single patient it suggests that functional heterogeneity in platelet

populations is associated with platelet age but that these differences may only be seen *in vitro* with subtle functional tests.

It has been observed that one of two convulxin (a collagen receptor agonist purified from Tropical rattlesnake *Crotalus terrificus* venom) binding sites on GPVI was lost from canine platelets as they aged *in vivo* and it was speculated that an extrinsic pressure on platelets is realised during senescence (Alberio *et al.*, 2002). Considering the recent spate of publications regarding the ability of metalloproteinase activity to mediate the shedding of GPIIb/IIIa and also GPVI (Bergmeier *et al.*, 2003; Bergmeier *et al.*, 2004; Bergmeier *et al.*, 2004) it is possible that the result obtained in the canine system reflects these *in vitro* data.

Megakaryocytes do not synthesise fibrinogen, therefore both megakaryocytes and platelets released into the circulation have to acquire the protein by endocytosis and storage within alpha granules. It is not surprising then to find that one group working with a canine model of platelet ageing has found a positive correlation between circulating platelet age and fibrinogen content (Heilmann *et al.*, 1994).

Examination of the packed volume of equal numbers of rat platelets isolated on the basis of density by centrifugation revealed that the densest platelets were approximately 2.4 times the volume of the light platelets (Karpatkin, 1972). The 'dense-large' platelets exhibited increased responsiveness to both thrombin and epinephrine (as assessed by ADP release) over and above the relative difference in volume and it was concluded that these platelets were the youngest ones in circulation. Interestingly, the dense-large

platelets contained 3.4 times the amount of glycogen and had six-fold greater rates of glycogenolysis and glycogenesis when compared to light-small platelets (Karpatkin and Charmatz, 1970). This data was suggested to be evidence that platelet ageing is associated with reduced metabolic function and reduction of carbohydrate reserves that would impact upon the activation process. These results were echoed by other groups who demonstrated that larger platelets isolated from human volunteers contained (on a per volume basis) more glycogen, ADP, ATP and surface sialic acid residues than smaller platelets (Carty and Gear, 1986) and that mean volume of platelets positively correlated with increased serotonin uptake and greater responses to agonists (Thompson *et al.*, 1982). However these authors found also that platelet volume did not correlate with platelet age, leading to the conclusion that megakaryocytes produce platelets of heterogeneous size, granule content and activatory abilities (Thompson *et al.*, 1983) and that larger platelets which would have a longer lifespan were 'more important' to haemostasis than smaller ones. Interestingly, large platelets, although more reactive to agonists such as ADP and thrombin, aggregated less rapidly than small platelets, a finding in accordance with a later study's finding (Carty and Gear, 1986).

An examination of over 7000 electron micrographs of rabbit platelets suggested that, after induction of thrombocytopenia by either neuraminidase or anti-platelet antibody treatment, newly made young platelets had very few dense granules and were less dense than older platelets (Mishory and Danon, 1978). The treatments used gave slightly different platelet loss kinetics; antibody treatment led to rapid thrombocytopenia (within 9 hours) whereas neuraminidase caused a slower rate of platelet clearance (48 hours). The platelets produced in response to antibody were larger than those produced after the

less acute thrombocytopenia caused by neuraminidase ($310 \pm 17.5 \mu\text{m}^2$ -vs.- $240 \pm 6.8 \mu\text{m}^2$, respectively). As platelet numbers recovered to normal levels the density of platelets increased along with an increased frequency of dense granules and loss of glycogen particles. The authors point out that the young-large platelets produced in their models may not represent a true reflection of normal megakaryocytopoiesis because of the suggestion that sudden losses of platelets leads to larger platelets being made, thus large platelet production could be a 'stress response'. The authors also point out that differences between young and old platelets may reflect increased degranulation of young platelets during collection and experimental procedure. These considerations notwithstanding, platelet senescence in the rabbit appears to be associated with at least increased density due to dense granule accumulation but decreased volume / size.

These contradictory results may infer that the processes and / or result of platelet senescence are species-specific. Data to support of this conclusion comes from a study that demonstrated that serotonin (5-Hydroxytryptamine (5-HT)) levels and platelet density positively correlate with each other in human platelets but the exact opposite is true in canine platelets (Pereira *et al.*, 1999).

The use of the nucleic acid specific stain thiazole orange (TO), has enabled the labelling of a 'young' population of platelets. After biotinylation the number of biotin-negative, TO-positive platelets rises from 0.72% at 30 minutes to 5.4% at 24 hours, indicating that TO stains newly synthesized platelets (Ault and Knowles, 1995; Dale *et al.*, 1995). These 'reticulated platelets' (RPs) constitute 6% and 7% of the canine and human total circulating platelet populations, respectively (Dale *et al.*, 1995; Rinder *et al.*, 1998).

The number of RPs (i.e. TO-positive) is dramatically increased from the normal level of ~4% to 42% in humans with immune thrombocytopenic purpura (ITP). This coincides with the observation that platelets in ITP compared to controls have an increased reactivity to the thrombin receptor agonist peptide (TRAP), suggesting that serious bleeding disorders are avoided in ITP by the lower activation threshold of the platelets (Rinder et al., 1998). However, large doses of TO can also label dense granules too and may give false results (Robinson *et al.*, 2000).

In summary, it is widely accepted that reduced adhesion to collagen and recalcitrance to agonists is associated with platelet senescence, whereas morphological changes appear to be more plastic and can be species specific. Thus, identification of older platelets on the basis of size, density, volume or numbers of dense granules has not been demonstrated unambiguously and data pertaining to differences between platelet populations isolated thus should be treated with great caution, especially in view of the probability that megakaryocytes produce platelets of morphological and biochemical heterogeneity.

1.10 Platelet Death and The Storage Lesion.

It is clear from studies performed several decades ago that platelets *in vivo* within organising arterial clots (Jorgensen et al., 1967; Nathaniel and Chandler, 1968; Davies et al., 1975) exhibit morphology similar to those generated *in vitro* (Brown *et al.*, 2000). They appear to be highly degranulated and were observed to have ‘gaps’ in their plasma

membrane, indicative of a necrotic end-point. Interestingly, the resolution of thrombus in a porcine model was associated with both the absolute degeneration of platelets and the increased occurrence of macrophages with what appeared to be phagocytosed platelets and fibrin within them (Jorgensen *et al.*, 1967). Further to these studies is the *in vitro* observation that, within 40 minutes of ADP-induced thrombus formation, leukocytes (primarily neutrophils, monocytes and eosinophils) had phagocytosed platelets (Shirasawa and Chandler, 1971). These observations suggest two things; that platelet death can be associated with haemostasis and that phagocytosis may be an important factor in the repair of vascular wounds.

Platelet storage lesion (PSL) is a collection of biochemical, morphological and functional changes that occur to platelets when stored *ex vivo* for the purpose of transfusion. PSL, along with bacterial contamination of platelet concentrates, is a major limiting factor in the shelf-life of platelet concentrates which is currently 5 days. Although noted several decades ago the PSL has received little attention apart from in the field of apheresis technology and platelet transfusion. Despite this fact a number of groups have, both explicitly and implicitly, observed and discussed the loss *in vitro* of platelet viability (Murphy and Gardner, 1971; Solberg *et al.*, 1986; Seghatchian and Krailadsiri, 2001; Bergmeier *et al.*, 2003; Wadhawan *et al.*, 2004).

PSL is characterised by loss of functional responsiveness of platelets to agonists; leakage of lactate dehydrogenase; glycolytic toxin accumulation (Bertolini *et al.*, 1993); loss of plasma membrane integrity (Murphy and Gardner, 1971); loss of ATP, platelet numbers and aggregate formation (Holme *et al.*, 1987); degranulation (Rinder and

Snyder, 1992); reduction of plasma pH; loss of CD42b (Michelson *et al.*, 1988) and lack of both recovery and survival upon transfusion of platelets into recipients (Rinder *et al.*, 2003).

Direct examination of apoptotic machinery has been performed by a limited number of groups. During storage of platelets over a 5-day period it was observed that both caspase-3 and caspase-9 proforms diminished concomitantly with increased amounts of their active subunits that were immunodetectable at 17kDa and 32kDa, respectively. Further analysis of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L indicated that although Bcl-2 levels remained constant Bcl-X_L levels decreased during storage at 37°C. The loss of viability according to and inability to reduce MTS correlated with the aforementioned changes to the apoptotic proteins, however none of these events could be inhibited by incubation of the platelets with pan-caspase inhibitor zVAD.fmk or the caspase-9 inhibitor zLEHD.fmk (Bertino *et al.*, 2003). However another group presented data contradicting the capsase-3 observation but confirming the capsase-9 data. It was also noted that PS was exposed on platelets during storage and that cytoskeletal proteins were degraded in a caspase-independent but calpain-dependent manner (Wadhawan *et al.*, 2004) similar to that seen during microvesiculation of freshly isolated platelets in response to the calcium ionophore A23187 (Wolf *et al.*, 1999).

The superficial similarities between PSL and apoptosis have led some authors to use the terms interchangeably with one and other (Leytin and Freedman, 2003) or discuss PSL in terms of an apoptotic program (Fratantoni, 1992), however it is arguable that PSL

results more from the accumulation of a metabolic toxin leading to necrotic lysis of platelets rather than an elegantly executed program of cell death.

1.11 Metalloproteinases and platelets.

Metalloproteinases are a family of zinc and calcium-dependent endopeptidases that have overlapping substrate specificities. They are characterised by N-terminal pre and pro enzyme domains that are cleaved to form active enzyme and a highly conserved catalytic domain connected via a hinge region to a hemopexin / vitronectin-like domain. They are grouped into three major sub-groups; collagenases (MMP-1, 8,13 and 18); gelatinases (MMP-2 and 9) and membrane type metalloproteinases (MMP-14, 15, 16, 17, 24 and 25). Their substrates are predominantly found within the extracellular matrix (ECM) protein laid down by cells, especially those associated with the vasculature. This links MMP activity with vascular remodelling and disease states such as cancer. Post-translational regulation of MMP activity is complex. Tissue inhibitors of metalloproteinases (TIMPs) bind to the conserved zinc-binding catalytic regions of MMPs to bring about inhibition of activity, thus the relative balance of MMPs and TIMPs determines the amount of MMP activity within a tissue (Kuzuya and Iguchi, 2003).

Platelets contain the gelatinases, MMP-2 and MMP-9 (Sawicki *et al.*, 1997); TIMP-4 (Radomski *et al.*, 2002) and release a trimolecular complex of MT1-MMP:TIMP-2:MMP-2 (Kazes *et al.*, 2000). The interplay between these proteins and their activities not only upon their known substrates but more subtle aspects of platelet function serves

to highlight the complexity of MMP regulation. Platelets release both proMMP-2 and proMMP-9 during activation, however it appears that MMP-9 is released more readily than MMP-2 in response to thrombin (Fernandez-Patron *et al.*, 1999). Immunogold studies of platelets revealed that MMP-2 (which colocalises with TIMP-4 (Radomski *et al.*, 2002)) is distributed throughout the cytosol of resting platelets and that activation in response to collagen led to release of the protein with retention of immunoreactivity upon the plasma membrane (Sawicki *et al.*, 1998).

1.12 Hexokinase and cell death.

Hexokinase is the first enzyme in the metabolism of glucose and catalyses the phosphorylation of glucose to form glucose-6 phosphate. Four distinct isozymes are described in humans (Wilson, 2003). Types I and II have a short membrane-integrating peptide sequence and are known to associate with the voltage dependent anion channel (VDAC) in the mitochondrial outer membrane (Mulichak *et al.*, 1998; Pastorino *et al.*, 2002) and also to other organelles such as the endoplasmic reticulum (Travis *et al.*, 1999). Therefore some hexokinase isoforms are intimate with proteins known to effect certain aspects of the cell death machinery. Type III has no such membrane spanning peptide but may be associated with the nuclear membrane as well as cytosol (Preller and Wilson, 1992), Type IV is known also as glucokinase and is the predominant form found in the liver.

In platelets the predominant form of hexokinase activity is that of type I, the majority of which is particle bound rather than cytosolic (Rijksen *et al.*, 1982). Impaired hexokinase

activity has been associated with decreased platelet activation in response to thrombin (Akkerman *et al.*, 1984) and a reduced glycolytic flux was observed in thrombin treated platelets from patients with Wiskott-Aldrich syndrome (Verhoeven *et al.*, 1989).

In favour of the hypothesis that growth factors are primarily involved with the support of glucose metabolism it was demonstrated that an IL-3-dependent cell line, upon growth factor withdrawal, rapidly down-regulated the expression of GLUT1, hexokinase II and phosphofructokinase (Vander Heiden *et al.*, 2001). This effect could be lessened by over-expression of the GLUT1 glucose transporter. The authors showed also that altering glycolytic flux by reducing exogenous glucose concentration caused Bax translocation to the mitochondria, release of cytochrome c to the cytoplasm and apoptosis.

A further link between glucose metabolism and apoptosis was emphasised by the observation that Bad, a pro-apoptotic member of the Bcl-2 family, could be regulated by glucose-dependent phosphorylation and exist as part of a pentameric protein complex along with protein phosphatase-1, Wave-1, PKA and glucokinase (Danial *et al.*, 2003). The authors of the report demonstrated that hypoglycaemia led to the dephosphorylation of Bad and cell death. Interestingly, Bad appeared also to affect glucose utilisation by modulating glucokinase activity, indicating that Bad, far from being just a harbinger of death, functions to maintain cellular homeostasis. It is likely that many proteins with functions in apoptosis will have appointed to them further functions distinct from their role in cell death.

1.13 Glucose metabolism in platelets.

Much of the pioneering work on metabolism in platelets was performed many decades ago. It is clear that platelets are metabolically active sub-cellular fragments. Early reports demonstrated that resting platelets require oxidative phosphorylation, however this is possibly not true considering the probability that platelets would have been activated by the harsh isolation conditions. The initial tethering of platelets to exposed areas of vasculature is likely to be energy-independent (Misselwitz *et al.*, 1987), subsequently, platelets up-regulate the surface expression of GLUT3, their sole glucose transporter (Sorbara *et al.*, 1997; Heijnen *et al.*, 1997) (fig. 1.6); an event that coincides with many energy-dependent processes such as integrin activation, degranulation and clot retraction (Holmsen *et al.*, 1974; Akkerman, 1978). It is now generally accepted that, at this energy-demanding time, platelets' ATP demand is met by oxidative phosphorylation with glucose being supplied by extracellular environment but also glycogen (Niu *et al.*, 1996). Although platelets appear to bind insulin and have insulin receptors (Hajek *et al.*, 1979; Kahn *et al.*, 1994) it is not clear what significance this ability has to platelet biology because GLUT3 is not known to be regulated by insulin.

1.14 Glycogen.

Mammalian platelets possess glycogen (Zingoni, 1952; Scott, 1967; du Plessis and Stevens, 2002; Field *et al.*, 2001). It is clear that glycogen is used up during the energy-demanding processes of activation and degranulation, but there have also been comments by some authors that glycogen content is reduced in what are believed to be older platelets (Ginsburg and Aster, 1972; Karpatkin, 1969; Karpatkin and Charmatz,

1970). The control of glycogen content in platelets has been examined in some detail due to the ease of isolation of platelets and their possession of functional glycogenic and glycolytic pathways. In contrast to muscle and liver glycogenolysis which is controlled by the phosphorylation and activation of glycogen phosphorylase by cAMP-dependent kinase PKA, platelets are thought to break down glycogen due to the activation of phosphorylase directly by calcium (Karpatkin et al., 1970; Gear and Schneider, 1975) and indirectly by calcium-calmodulin (Gergely *et al.*, 1980). As with all metabolic pathways, none exist in isolation and glycogenesis appears to be concomitantly down-regulated by increased intra-platelet calcium levels that inhibit glycogen synthesis. Thus platelets represent an unusual PKA-independent / calcium-dependent system of glycogen metabolism that is distinct from that seen in muscle and liver. Another key regulator of glycogen metabolism recently identified in platelets is Glycogen Synthase Kinase 3 (GSK3) (Barry *et al.*, 2003). The un-phosphorylated enzyme (present in platelets as both GSK3 α and GSK3 β) phosphorylates glycogen synthase to inactivate it thereby shifting the equilibrium in cells towards glycogen breakdown. The effects of GSK3 can be inhibited by lithium's ability to block the dephosphorylation of GSK3 (fig. 1.7) and maintain it in an inactive form (Zhang et al., 2003; Chalecka-Franaszek and Chuang, 1999); thus lithium might be expected to inhibit glycogen depletion in platelets. In platelets inactivation of GSK3 is caused by phosphorylation by PI-3 Kinase during platelet activation in response to collagen or thrombin. At first sight this appears to be counter intuitive, but may be indicative of an uncoupling of GSK3 from glycogen metabolism activity in platelets during activation. GSK is known to have varied effects within cells and has been implicated in the regulation of apoptosis; responses to amino

acid starvation; the development of cancer and metabolic diseases such as diabetes (Frame and Cohen, 2001).

Caffeine and a novel caffeine-based drug CP-91149 are both effective inhibitors of glycogen phosphorylase (fig. 1.7). Although structurally similar their mode of action is different because they can act synergistically to bring about a reduction in phosphorylase activity *in vitro* and it is believed they target distinct sites upon the enzyme that affect enzyme-substrate interactions. Despite inhibiting phosphorylase, caffeine has broad pharmacological effects on cellular processes. It can affect adenosine receptor expression on platelets by initially causing down regulation with concomitant recalcitrance to agonists followed by massive up-regulation of receptors with resultant hypersensitivity to agonist (Varani et al., 1999; Paul et al., 1993). CP-91149 is a novel drug developed by Pfizer for the control of hyperglycaemia in diabetics (Martin *et al.*, 1998). It offers a lower dose inhibitory effect than caffeine (μM versus mM ranges, respectively) and is effective *in vivo* at lowering hyperglycaemia in diabetic *ob/ob* mice without causing hypoglycaemia (Martin *et al.*, 1998) and is known to concomitantly activate glycogen synthase (Aiston *et al.*, 2001).

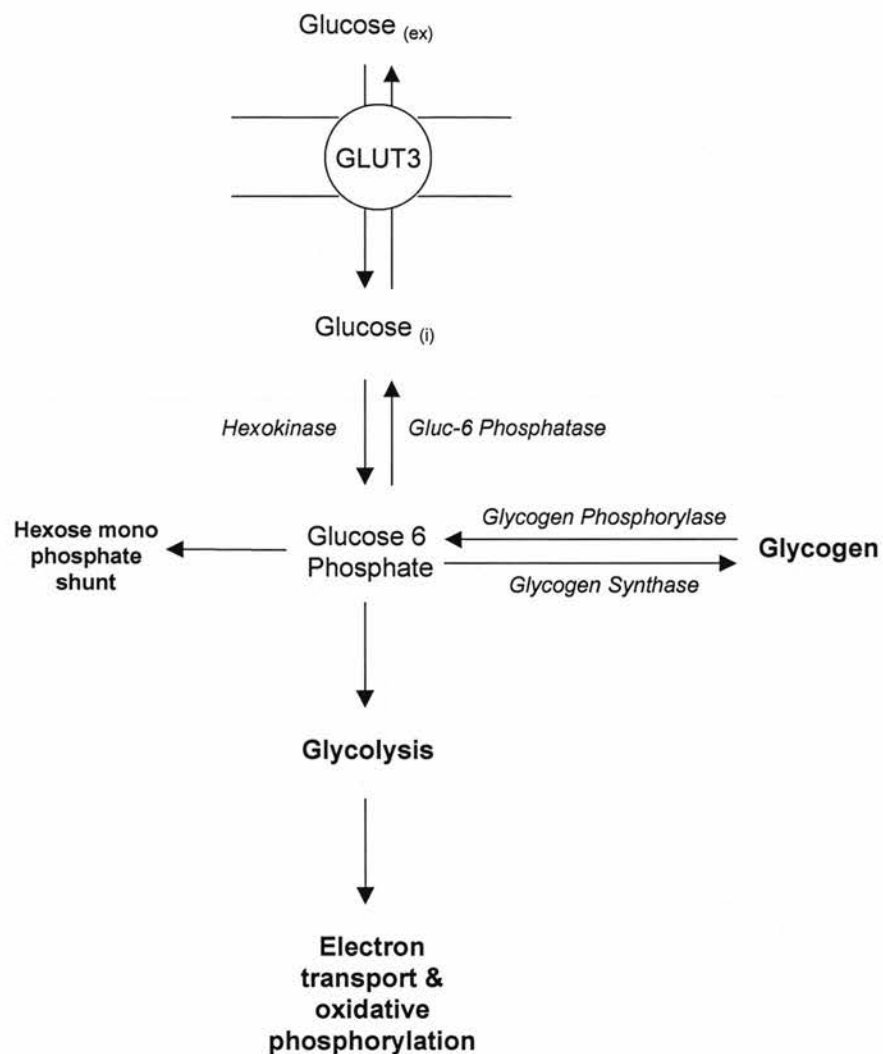


Figure 1.6. Metabolism in platelets.

A very brief overview of the major glycolytic and glycogenolytic pathways within platelets. Platelets are thought to possess the insulin independent glucose transporter GLUT3, which can be up-regulated upon their surface during activation. Transported glucose is phosphorylated by hexokinase activity and is then bound for glycogen synthesis, glycolysis and hexose monophosphate shunt.

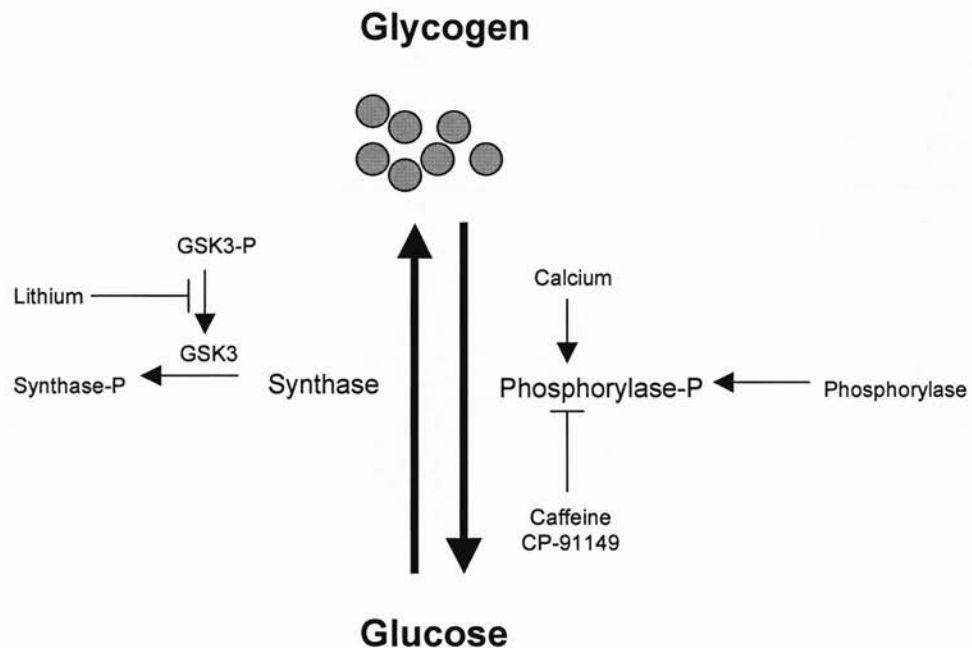


Figure 1.7. Glycogen metabolism.

Glycogen metabolism is regulated by both synthase and phosphorylase activities. Inactivation of synthase occurs due to phosphorylation by glycogen synthase kinase 3 (GSK3), whose activity is inhibited by phosphorylation. Catabolism of glycogen is catalysed by phosphorylase, whose activity is increased by binding calcium and / or by phosphorylase kinase activity. Both caffeine and a novel caffeine analogue CP-91149 are able to inhibit phosphorylase activity and prevent glycogen breakdown, whereas a catabolic equilibrium is prevented by increased synthase activity evoked by lithium.

1.15 Autophagy.

Although the presence in platelets of caspase 3 has been described by numerous authors (Shcherbina and Remold-O'Donnell, 1999; Brown et al., 2000) and activity of the pro-form suggested by others (Piguet *et al.*, 2001) there is no clear correlation between the activity and *in vitro* death nor inhibition of its activity and platelet survival (Bertino et al., 2003; Wadhawan et al., 2004). Therefore, it appears that platelets die *in vitro* independently of caspase activation (Brown et al., 2000; Wadhawan et al., 2004). Autophagic cell death is persistently associated with vacuolisation and destruction of cytoplasmic contents including organelles leading to near complete catabolism and a necrotic-like end point (Thummel, 2001; Lee and Baehrecke, 2001; Bera et al., 2003).

Apoptosis is the best understood means of programmed cell death but it is not the only one (Bursch et al., 2000; Lockshin and Zakeri, 2002; Lockshin and Zakeri, 2004). The identification of apoptosis relies, in part, on caspase-dependent changes (Samali *et al.*, 1999) to nuclear morphology (chromatin condensation and nuclear body formation (Kerr *et al.*, 1972) - something that can clearly not be done with platelets. Thus, apoptosis by any classical description can never be securely appointed as the program of death used by platelets.

The alternatives to apoptosis are not well described but involve both apoptotic and necrotic-like end points that appear to be elicited by catastrophic changes in lysosomal integrity (Boya *et al.*, 2003) or extensive autophagic destruction of cytosol and

organelles (Yuan *et al.*, 2003). Interestingly these changes appear not only in mammalian cells but those of lower species such as *Drosophila melanogaster* (Thummel, 2001) and the single-celled protozoan parasite *Leishmania donovani* (Lee *et al.*, 2002) and therefore represent forms of cell death that, in evolutionary terms, preceded apoptosis.

Autophagy is the bulk degradation of cytosol and organelles within the lysosomes and is a constitutive process required for the homeostasis of organelles and protein complexes. It is a process that can be up-regulated in response to glucagon (Deter *et al.*, 1967) and upon nutrient deprivation (Bergamini *et al.*, 1994) and is regarded as an important adaptive response that allows for the recycling of essential amino acids from non-essential cellular components and the rapid liberation of glucose from glycogen stores. Although autophagy is associated with cell death it has not been defined clearly as the cause or consequence of it (Bursch *et al.*, 2000; Thummel, 2001). However, a very recent report discusses the first genetic evidence that genes important for autophagy can be, under certain circumstances, required also for the execution of cell death (Yu *et al.*, 2004).

The identification of autophagy has historically relied upon the observation at the electron microscopic level of multi-lamellar bodies surrounding identifiable cellular components (Ericsson, 1969). Work with the yeast *Saccharomyces cerevisiae* identified 17 genes necessary for the induction of autophagy in response to nitrogen deprivation (Tsukada and Ohsumi, 1993). Of particular importance to the field has been the identification of the pre-autophagosomal structure (Suzuki *et al.*, 2001) whose

formation precedes and is necessary for the formation of an autophagic vacuole (Kirisako *et al.*, 1999). One of the five ATG gene products that comprise the PAS, Atg8p (a ubiquitin-like protein known as MAP1-LC3 or LC3 in mammalian cells (Kabeya *et al.*, 2000) appears to be unique to the autophagy machinery and its accumulation is now used widely as a marker of autophagy not only in yeast but also *Dictyostelium* (Otto *et al.*, 2003) and mammalian cells (Tanida *et al.*, 2002; Asanuma *et al.*, 2003).

Although autophagy is becoming less enigmatic as a cell biological process there are sub-types of lysosomal / autophagic processes that remain poorly understood. Most work has dealt with *macro*-autophagy, which is characterised by the *de novo* synthesis of double membrane around target organelles / cytosol, but there exists also micro-autophagy, chaperone-mediated autophagy (CMA), micro and macro-pexophagy (Huang and Klionsky, 2002) and crinophagy (Dunn, 1994).

Micro-autophagy is characterised by the protrusion of the lysosomal membrane around and the engulfment of local cytoplasm / organelles. Interestingly, this form of autophagy is induced after the yeast *Pichia pastoris* is grown on methanol rather than glucose. Peroxisomal enzymes are synthesised for the usage of methanol, however when the yeast is transferred to glucose these enzymes and the peroxisomes are selectively degraded within the yeast vacuole by a process of micro-autophagy (Yuan *et al.*, 1997; Yuan *et al.*, 1999). Both pexophagy and micro-autophagy represent similar systems of organelle destruction within the lysosome. Chaperone mediated autophagy (CMA) relies on the presence within a target protein of the consensus sequence KFERQ

which is recognised and bound by the cognate heat shock protein hsc73 (Dice *et al.*, 1990) the complex binds to the lysosome, a process apparently requiring cleavage of Lamp-2 at the lysosomal membrane by Protective Protein / Cathepsin A (PPCA), and then degraded (Cuervo *et al.*, 2003). CMA is important in the destruction of certain annexins (annexins II and VI but not V and XI) in response to serum starvation (Cuervo *et al.*, 2000) and it is noteworthy that approximately 40% of the enzymes involved in glycolysis have KFERQ-like sequences.

Crinophagy is probably the least well understood but yet one of the most important forms of autophagy in mammalian cells. The process involves the fusion of storage granules directly with lysosomes and is an important mechanism maintaining granule homeostasis in highly secretory cells such as the insulin secreting β -cells in the pancreas (Koike *et al.*, 1982), hepatocytes (Lenk *et al.*, 1991), prolactin-secreting mammatrophs (Bernabe *et al.*, 2001) and neurons (Boudier and Picard, 1976).

Although platelets have lysosomes it is not known what role they play in platelet biology or haemostasis. Autophagic-like structures have been observed in circulating platelets from patients with carcinoid syndrome (Lewis *et al.*, 1976) and a patient with Danon's disease (a lysosomal storage disease with normal acid maltase activity), was noted to have both abnormal platelet function and accumulation of glycogen (Katsumi *et al.*, 1994). Therefore a precedent for autophagy with abnormal platelet function / pathology is present in the literature. It is not known whether autophagy has any role in the death of platelets and this form of cell death is examined in this thesis.

1.16 Assessment of platelet viability.

One reason for the scant information regarding platelet death is that few markers are available that allow for the rapid, objective discrimination of live from dead platelets. Their anucleate nature prevents analysis of plasma membrane integrity with DNA-interchelating dyes and their tiny size precludes the use of vital stains such as trypan blue complemented with microscopy for scoring viability. While the viability of whole platelet populations can be routinely assayed for by examining the release of lactate dehydrogenase (LDH) (Holme et al., 1987; Bode and Miller, 1989; Bertolini et al., 1993), release of annexin-V (Krailadsiri *et al.*, 1997) and changes in pH (Bertolini *et al.*, 1993) such data does not allow the isolation and study of individual platelets (Solberg et al., 1986; Li et al., 2000). Ultrastructural identification of 'lysed and balloon' platelets has been used to evaluate the frequency of dead platelets in stored platelet concentrates (PC) (Murphy and Gardner, 1971; Solberg et al., 1986) but due to the obvious expense and labour intensity of such work a marker of viability that can discriminate live from dead platelets is highly desirable. Also, any such marker should be amenable for inclusion into experiments involving live and dead platelets without having deleterious effects on platelet biology.

1.17 Summary and Overall Aims of Research Program.

Platelet populations display biochemical and morphological heterogeneity but it is unlikely that this reflects age-related differences between platelets and is more likely a result of the inconsistencies of platelet production. Platelet storage lesion has been examined in some detail at the level of whole platelet populations or in the context of transfusion recovery success and lifespan but reports examining the phenotype of individual platelets, the surface changes that occur as a result of PSL or the factors dictating the onset of PSL are scant. Annexin-V, a marker of cell death that has been used to demonstrate platelet death is also widely exploited to demonstrate changes to membrane asymmetry during activation in viable platelets.

- An initial step in this program of work is the confirmation and subsequent confident identification of platelet death. This is to be performed by careful morphological and functional analysis of unwashed platelets stored at 37°C.
- Secondly, a marker that allows the rapid discrimination of live from dead platelets is to be sought.
- Thirdly, changes to the surface phenotype of dead platelets are to be studied.
- Fourthly, the mechanism of platelet death is to be examined.
- Lastly, it is important to understand why platelets are dying *in vitro*, therefore factors controlling their survival are to be explored.

2 MATERIALS AND METHODS.



2.1 Reagents and Antibodies.

All stock chemicals were from Sigma unless otherwise stated. Adenosine diphosphate (ADP), Formaldehyde, carbonyl cyanidem-chlorophenylhydrazine (CCCP), Hanks buffered saline solution (HBSS), Dulbeccos modified Eagle's Media (DMEM), prostacyclin (PGI₂), Calcium ionophore (A23187) were all from Sigma (Dorset, UK); Calcein-acetoxymethyl ester and *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide (FM4-64); R-Phycoerythrin and Alexa⁴⁸⁸-conjugated Streptavidin; LysoTracker Red; Mitotracker Green; all Alexa-conjugated antibodies and Alexa⁴⁸⁸-conjugated fibrinogen were from Molecular Probes (Oregon, USA); Convulxin was from Pentapharm (Switzerland); Thrombin activated agonist peptide (TRAP), Fibrillar Collagen solution was from Chronolog (Haverton, USA), GM6001 and Dimethyl Sulphoxide (DMSO) were from Calbiochem (California, USA). R-Phycoerythrin conjugated anti-human CD62P was from DakoCytomation (Cambridgeshire, UK); R-Phycoerythrin anti-human CD42b, clone HIP1, was from BD Pharmingen; Annexin-V conjugated to either FITC or RPE, FITC-conjugated anti-Human α_{IIb} was from Caltag Laboratories (California, USA). Sterile sodium citrate solution (Phoenix Pharma (Gloucester, UK). Anti-human Lamp-2 antibodies (H4B4) were supplied as a partially purified IgG supernatant from the University of Iowa Hybridoma Bank.

2.2 Blood collection from humans.

Whole blood (40ml) was collected into 50ml plastic Falcon tubes containing 4ml of 3.8 % sodium citrate from healthy human volunteers. Blood was centrifuged at 350 x g for

20 minutes in a swing-out rotor centrifuge. The resultant upper layer of platelet-rich plasma was removed to a fresh 50ml capped plastic tube and maintained at room temperature or at 37°C until processed for experimentation. A further aliquot was removed to a 500µl Eppendorf, maintained at 37°C and used for estimation of platelet numbers using a Coulter counter.

2.3 *In vitro* platelet culture.

Platelets were cultured at 37°C or room temperature unless otherwise stated. Platelets were cultured as citrated platelet rich plasma (cPRP); as a washed suspension (resuspended in calcium-free Hanks buffered saline solution (HBSS): MgSO₄ 0.5g/l; KCL 0.4g/l; KH₂PO₄ 0.06g/l; NaHCO₃ 0.35g/l; NaCl 8g/l; Na₂HPO₄ 0.048g/l)) or as a 50-fold dilution of PRP. This dilution factor was chosen because it allowed platelets to be analysed by flow cytometry without causing blockages caused by large aggregates that formed during culture or activation. D-Glucose was added to a final concentration of 5mM unless otherwise stated. 2ml or 0.5ml capped Eppendorf tubes were used as culture vessels and were filled to capacity with platelet suspension. Cultures were maintained in a water bath for the required periods of time as stated in the text with twice daily inversion.

2.4 Platelet Aggregometry.

A gross measure of platelet function can be determined by increased transmission of light through stirred platelet rich plasma challenged with agonists. The initial stages of platelet activation involve cytoskeletal rearrangements that transform resting discoid

platelets into irregularly shaped entities. This 'shape change' causes less light to pass through the platelet suspension and is seen as an increase in absorbance on aggregometry plots. Platelet activation proceeds with the switching of low affinity adhesion molecules into their high affinity state; most importantly is the $\alpha_{IIb}\beta_3$ integrin complex, this complex binds fibrinogen (amongst other proteins) that acts as a bridging molecule between activated platelets. As the frequency of platelet-platelet interactions increases light passes more easily through the platelet suspension and increased light transmission is recorded. High order activation causes degranulation, a process of granule secretion and surface expression of P-Selectin (CD62P). Granule exocytosis leads to increased extracellular ADP and serotonin that positively feedback to platelets in autocrine and paracrine fashion to promote further activation and aggregation. Maximal aggregation is set as the amount of light passing through a platelet-free solution.

Although widely used for the assessment of platelet function it is important to mention the limitations of aggregometry. Plasma fibrinogen will be cleaved by thrombin to form fibrin. Fibrin meshes will trap within themselves any particles, be they platelets or inert beads. Aggregation can appear to proceed towards maximal levels but in this circumstance is not a reflection of platelet function but only of thrombin's effects on fibrinogen. If thrombin is to be used as agonist platelets must be first washed from plasma and resuspended in a fibrinogen free solution. Evidence of platelet function where thrombin has been used as agonist and platelets were within plasma should therefore be treated with great caution (Hoffmeister et al., 2003). Thrombin analogues such as thrombin receptor agonist peptide (SFLLRN / TRAP) obviate these problems,

as they have no enzymatic effect on fibrinogen but still stimulate platelet Protease Activated Receptors (PARs) (Hui *et al.*, 1992).

Temperature has also a profound effect on platelets' response to agonists. Reducing the temperature below 37°C primes platelet responses so that their resting state is lost and they aggregate more readily than if at 37°C. Aggregometer chart recordings of colder platelets invariably show a reduced increased absorbance of light due to the platelets already having shape change due to lower temperatures (i.e. they are in 'primed' state) (see Appendix fig. 8.1).

All procedures were carried out in a Chronolog dual chamber aggregometer at 37°C with temperature-equilibrated equipment and platelets unless stated otherwise. 500µl of PRP or washed platelet suspension was added to siliconised glass aggregometry tubes containing a steel stir-bar. Basal and maximal light transmissions were set with un-activated platelets and platelet poor suspensate, respectively. Platelets were stirred at 1000 rpm, calcium was added back to 2mM and then agonist was added. On occasion platelets were pre-incubated with pharmacological agents for times specified in the figures. Aggregation proceeded for times as specified in the figures (from 2 minutes to 20 hours).

2.5 Platelet activation.

Platelets were activated with various agonists (ADP; Convulxin; Thrombin; Thrombin receptor agonist peptide (TRAP); A23187) in the presence or absence of 2mM calcium

chloride and / or other treatments as described in the text. Activation was allowed to proceed with or without agitation for 10 minutes at 37°C whereupon the reaction was stopped by the addition of 1 volume of 2% formaldehyd in HBSS A or 10-fold dilution of platelets in HBSS if live staining was subsequent to activation. For analysis of the effects of Antimycin on platelet activation platelets were pre-incubated with or without 10µg/ml Antimycin for 1 hour prior to activation.

2.6 *In vitro* Biotinylation of Platelet Surface Proteins.

PRP was incubated for 5 minutes with 0.1µg/ml prostacyclin then centrifuged at 1000 x g for 5 minutes. The platelet pellet was resuspended in HBSS and incubated with 100µM NHS-Biotin for 30 minutes at 37°C in order to label exposed amine groups on the platelet surface. Platelets were washed from the biotinylation solution as described and resuspended in autologous platelet-poor plasma (prepared by centrifuging PRP at 13.5K rpm for 1 minute at room temperature). Biotinylation was confirmed by staining platelets with fluorescently-conjugated streptavidin (see below).

2.7 *In vivo* biotinylation of murine blood cells.

The aging of a platelet population can be observed *in vivo* by biotinylating whole blood and examining biotin-positive platelets at subsequent time points. Two methods allow for the labelling of platelets one involves the infusion of biotin directly into the bloodstream of the animal the other requires platelets to be removed, labelled, washed and transfused back into animals. After blood sampling the biotinylated platelets can be identified by their ability to bind fluorescently conjugated streptavidin. The method is

simple and has been employed in a variety of animal models e.g. dogs, mice rabbits and monkeys (Dale et al., 1995; Peng et al., 1994; Berger et al., 1998; Michelson et al., 2001; Franco et al., 1994). Although there is widespread acceptance of the technique others have reported an impaired ability of biotinylated human platelets to aggregate (Magnusson *et al.*, 1998).

Utilisation of this method has demonstrated that older platelets have an impaired response to thrombin (as evidenced by the 1.78-fold increase in the EC₅₀ required to induce surface expression of P-Selectin) (Peng *et al.*, 1994) that platelets accumulate fibrinogen but not IgG as they age (Heilmann *et al.*, 1994) and that platelet clearance, at least in the mouse, is independent of CD62P (Berger *et al.*, 1998). Interestingly, although flow cytometry collects information regarding cells' refractivity and defractivity (roughly translating as volume and granularity) no one has published any data suggesting there are changes in these characteristics as platelets age *in vivo*, suggesting that the changes to volume, dense granule and glycogen content associated with aging have not been noticed by flow cytometry, or that these changes are not as is currently believed due to senescence related processes.

The functional consequences of platelet biotinylation have been debated. Some researchers have found that after *in vitro* labelling the aggregation of platelets in response to 10μM ADP is impaired (Magnusson *et al.*, 1998). However, this contrasts other reports that suggest this is not the case (Heilmann *et al.*, 1993; Ault and Knowles, 1995).

NHS-Biotin was diluted with DMSO to a stock concentration of 40mg/ml. The stock was diluted immediately prior to injection 4mg/ml with sterile saline or PBS. It is important to note that the efficacy of biotinylation relies on rapid usage of this solution, once the NHS-Biotin is introduced to water the succinyl group hydrolyses and free biotin is liberated. This reaction is thought to last only a few minutes. The liberated biotin reacts with free amine groups in its vicinity and is stable for many days. Mice were injected intravenously (i.v) with 150µl of 4mg/ml NHS-Biotin (a total of 600µg of NHS Biotin per mouse). A second injection was performed 1 to 4 hours later in order to label any platelets that were sequestered within the spleen during the previous injection. This method of biotinylation labels all circulating blood cells and is stable for the duration of the platelets lifespan as assessed by its persistence upon red blood cells without any loss of signal. One caveat that must be mentioned for this method is that plasma proteins are also biotinylated and any cells using / accumulating such plasma proteins on their surface will show a persistent signal when stained *ex vivo* with biotin-specific probes.

2.8 Incubation of platelets with Annexin V.

Annexin-V is a member of a large family of proteins known to negatively affect coagulation due to their ability to compete with Factor Xa for exposed PS residues on platelets (Thiagarajan and Tait, 1990). Therefore, although annexin-V binds to PS exposed as a consequence of programmed cell death and necrosis, its use as a marker of platelet viability is limited because of its ability to interact with viable platelets during the shedding of microparticles (Stuart *et al.*, 1995). Thus annexin-V positive platelets

can be securely classed as dead only when corroborating phenotypic information at the ultrastructural level is presented (Brown *et al.*, 2000). It is important to note also that the mechanism of PS exposure used during platelet activation appears to be distinct from that used for PCD, thus annexin-V binding to platelets is not a *bone fide* marker of their death (Martinez and Freyssinet, 2001). Its use with platelets also requires millimolar levels of calcium that lead to calcium influx into platelets and activation when store-mediated calcium entry / capacitative calcium entry has been initiated (Rosado and Sage, 2000; Dobryднева and Blackmore, 2001). Thus, there are limitations to its usefulness for experimentation with mixed populations of viable and non-viable platelets when low calcium levels are required for maintenance of the resting state.

Platelets were incubated for 5 minutes on ice with a 1:100 dilution of either FITC-conjugated or RPE-conjugated Annexin-V in the presence of 2mM calcium chloride. The platelet sample was then diluted 100-fold with cold calcium-free HBSS and immediately analysed for green fluorescence by flow cytometry in a Coulter XL benchtop flow cytometer using unstained platelets as an autofluorescent control. Annexin-V binding to platelets was discernible as a single log order (ten-fold) shift in fluorescence signal. A widely used positive control for the assay are platelets that have been activated in the presence of calcium ionophore A23187, which is known to cause dramatic shedding of microvesicles and exposure of PS.

2.9 Examination of platelet cardiolipin with 10-N-nonyl acridine orange.

Nonyl acridine orange associates with cardiolipin (a phospholipid found exclusively in mitochondria) and is thought to associate with mitochondria independently of their energy state / membrane potential (Maftah *et al.*, 1989). Therefore the dye has been useful for the determination of mitochondrial biogenesis during cell cycle progression (Leprat *et al.*, 1990) estimation of mitochondrial mass (Pieri *et al.*, 1993) and changes to mitochondrial cardiolipin levels during aging (Maftah *et al.*, 1994). Its use as a marker of early cell death events involves its ability to detect the oxidation state of cardiolipin (Nomura *et al.*, 2000). The oxidation of cardiolipin, reduction of its synthesis or interaction with caspase 8-cleaved tBid have all been shown to precede the release of cytochrome c into the cytosol (Asumendi *et al.*, 2002; Petrosillo *et al.*, 2001; Shidoji *et al.*, 1999; Nomura *et al.*, 2000; Ostrander *et al.*, 2001; Lutter *et al.*, 2000; Lutter *et al.*, 2001) suggesting that alteration of cardiolipin is an important and early determinant of programmed cell death. Thus the inability of a cell to accumulate NAO is indicative of changes to cardiolipin that may reflect either oxidation of the phospholipid or complete loss of mitochondria. Caution is required when using NAO due to the fact that it is sensitive to mitochondrial membrane potential, however it is not known if its failure to be retained within mitochondria that have lost their membrane potential is due solely to changes in $\Delta\Psi_m$ or due to concomitant changes to the biochemistry of cardiolipin (Jacobson *et al.*, 2002).

Platelets as a 1:500 dilution of PRP or washed concentration were incubated with 500pM solution of NAO for 10 minutes at 37°C (a period of time that allowed maximal loading of the dye as assessed by flow cytometry; see appendix fig. 8.3). Platelets were

then analysed by flow cytometry for associated green fluorescence, which was two log orders greater (100-fold) than unstained controls. For microscopy platelets were incubated as a 1:50 dilution of PRP or washed platelets with 5nM NAO for 10 minutes at 37°C. An aliquot of the sample (8µl) was then spotted onto a glass microscope slide and gently cover-slipped. Excess fluid was removed using the corner of a tissue and platelets were immediately viewed by epifluorescence microscopy.

2.10 Analysis of platelet viability using calcein-AM.

Calcein-acetoxymethyl ester is a non-fluorescent compound related to fluorescein that is permeable to biological membranes. Diffusion of calcein-AM across the plasma membrane introduces it to intracellular esterase activity that liberates free calcein, a highly fluorescent and membrane-impermeable compound that is thus trapped within the cell. Thus, the accumulation of calcein fluorescence within a cell relies on two properties, esterase activity for the hydrolysis of the AM ester from calcein in order to liberate free fluorescent calcein and an intact plasma membrane for the retention of the free calcein. An inability to accumulate this dye is therefore indicative of cell death relating either to loss of plasma membrane integrity or loss of esterase activity. As such, calcein-AM is a useful marker of cellular viability and has been used to study cytotoxic killing by T cells (Wang *et al.*, 1993) the viability of Schwann cells (Decherchi *et al.*, 1997) and the detection of early apoptotic events in PC12 and NIH3T3 cells (Gatti *et al.*, 1998). Although calcein has been used to label platelets for *in vivo* tracking of thrombus formation it has not been used as a viability marker (Denis *et al.*, 1998).

For flow cytometric analysis of calcein-AM accumulation by platelets cPRP or washed platelet suspension was diluted 500-fold in calcium free HBSS containing 100nM calcein-AM, incubated for 10 minutes at 37°C and then analysed for associated green fluorescence. Calcein-AM did not significantly accumulate in platelets beyond the ten-minute time-point. Preliminary experiments indicated that 100nM calcein led to at least a three hundred-fold increase in green fluorescence beyond the platelets' auto-fluorescence. A similar (10-fold more platelets and 10-fold more calcein-AM) methodology was followed for microscopic analysis of calcein accumulation.

2.11 Assessment of viability using FM4-64.

N - (3 - triethylammoniumpropyl) - 4 - (p-diethylaminophenyl - hexatrienyl) pyridinium dibromide (FM4-64) is a lipophilic sytryl dye that has been used to label the vacuole in yeast. It inserts into the plasma membrane and by an endocytic process that is both energy and time dependent is trafficked to the vacuole (Vida and Emr, 1995). It has been used with mammalian cells to specifically label the lysosomes of neutrophils during phagocytosis of opsonised red blood cells (S. Grinstein pers comm. / unpublished results) and to track exocytosis of synaptic vesicles from neurons (Fernandez-Alfonso and Ryan, 2004). Due to independence of energy and temperature for the initial insertion of the dye into plasma membranes but the dependence upon energy, temperature and time for its trafficking, it was hypothesised that it may be useful for differentiating viable from dead platelets by virtue of its maintenance at the plasma membrane of dead cells but its endocytosis into viable cells. Thus when washed

from the dye dead cells would have bright plasma membranes and viable cells would have bright lysosomes.

For flow cytometric analysis of FM4-64 accumulation by platelets cPRP or washed suspension were diluted 500-fold in calcium free HBSS containing 5 μ M FM4-64, incubated for 10 minutes at 37°C and then analysed for associated red fluorescence by flow cytometry or microscopy.

2.12 Analysis of platelet mitochondrial membrane potential using JC-1.

A mitochondrial stain used widely in the field of cell death research is 5,5',6,6' - tetrachloro - 1,1',3,3'- tetraethylbenzimidazolyl - carbocyanine iodide (JC-1), a membrane permeable organic cation that accumulates within the matrix of respiring mitochondria (Smiley *et al.*, 1991). Within the matrix JC-1 monomers stack to form polymers, a process known as J-aggregation. Monomeric JC-1 emits fluorescence in the green wavelength whereas stacked JC-1 emits red fluorescence. This change in fluorescence emission qualities allows for the ratiometric analysis of green to red fluorescence and is a measure of mitochondrial membrane potential ($\Delta\Psi_m$) (Cossarizza *et al.*, 1993). Treatment of cells with protonophores that disrupt $\Delta\Psi_m$ leads to the loss of matrix JC-1 accumulation, consequent loss of red fluorescence and an increase in green fluorescence. It is important to note that uptake by cells of JC-1 (like other organelle-accumulating dyes) is dependent upon both $\Delta\Psi_m$ and plasma membrane potential ($\Delta\Psi_p$), thus cells with intact plasma and mitochondrial membrane potentials have both red fluorescing mitochondria but also green fluorescence associated with

cytoplasm. Loss of $\Delta\Psi_m$ but maintenance of $\Delta\Psi_p$ therefore leads to reduced mitochondrial-associated red fluorescence and increased cytoplasmic green fluorescence. A number of caveats have arisen in the past few years regarding use of this dye. It is thought that J-aggregation can occur spontaneously in solution and that these aggregates can associate with cells yielding false positive results (*pers. comm.* From the NIH List). It has been found that results gained with JC-1 and other, better characterised, $\Delta\Psi_m$ -sensitive dyes such as tetramethylrhodamine do not necessarily correlate with each other.

Platelets as 1:500 dilution of PRP or washed platelet suspension were incubated with JC-1 solution (1 μ M) for 20 minutes at 37°C and immediately examine by flow cytometry. Platelets with intact mitochondrial membrane potential display dual green and red fluorescence whereas those without fail to accumulate JC-1 to their mitochondrial matrices and thus do not contain red-fluorescing J-aggregates; such platelets display higher green to red ratio of fluorescence. Gating on high red / low green versus low red / high green platelets is discriminatory for platelets with and without intact $\Delta\Psi_m$, respectively (Cossarizza *et al.*, 1993).

2.13 Examination of intraplatelet calcium (calcium_(i)) with FLUO3.

Fresh platelets or platelets stored *in vitro* for 24 hours were incubated as a 1:500 dilution of cPRP (in HBSS) for 30 minutes at 37°C with Fluo-3 at a final concentration of 1 μ M. Platelets were then analysed for green fluorescence using a Coulter XL bench-top flow cytometer. Unstained platelets were used to establish autofluoresence, which

was set to within the 1st log order of brightness. Increases in calcium_(i) are detected as an increase in green fluorescence which can be invoked by addition of agonists to platelet suspensions. Results were expressed as mean fluorescence units (mfu).

2.14 Quantification of mitochondria within platelets.

Platelets were incubated for 30 minutes at 37°C in the presence of 100nM Mitotracker green and / or 200nM LysoTracker red. Platelets were allowed to spread on glass coverslips for 10 minutes. Platelet mitochondria were seen as individual punctate structures and do not share the 'string of beads' morphology characteristic of other cell types. At least 50 platelets from sequential, non-overlapping fields of view were quantified for the number of mitochondria.

2.15 Electron Microscopy.

Platelets in platelet rich plasma were fixed with an equal volume of 3% glutaraldehyde in 0.1M sodium cacodylate buffer for at least 1 hour at 37°C. Fixed platelets were pelleted, washed and processed for transmission electron microscopy with osmium tetroxide, lead citrate, araldite embedding and 60nm sectioning. Samples were analysed on a Philips CM12 transmission electron microscope.

2.16 Periodic Acid Schiff (PAS) staining of platelets.

Platelets were fixed with 1 volume of 2% formaldehyde at 37°C for 30 minutes, washed from fixative by centrifugation at 500 x g for 5 minutes and resuspension in ½ volume

of 0.5% periodic acid for 5 minutes at ambient temperature. Platelets were rinsed from the acid solution by two washes (as described) with HBSS. The platelets were then incubated with Schiff reagent ($\frac{1}{2}$ original platelet volume) for up to 15 minutes or until a colour change could be seen by eye. Platelets were washed from the reagent by one wash in HBSS, resuspended in $\frac{1}{2}$ volume of HBSS. An aliquot of platelets was then spotted onto a glass slide, cover-slipped and viewed under phase microscopy. Fields of glycogen can be seen as red-purple bodies within megakaryocytes (Ogura *et al.*, 1985) and platelets (Woo *et al.*, 2002).

2.17 SDS PAGE and Western Blotting.

Proteins from equal number of platelets per donor were lysed by boiling in Laemmli buffer A. For analysis of hexokinase washed platelets in H₂O were sonicated 5 times for 10 seconds each on ice to bring about incomplete destruction of membranes so that a hard centrifugation step performed at 13,000 rpm for 30 minutes at 4°C separated membrane (pellet) from cytosolic fractions (supernatant). The respective fractions were placed in one volume of Laemmli buffer A (for cytosolic fraction) or further treated with 1% Triton-X 100 in 100µl of PBS for 15 minutes on ice in order to liberate proteins from membranes. Triton-treated pellet was re-centrifuged to obtain a supernatant containing triton-solubilised proteins to which was added 1 volume (90µl) of Laemmli buffer A.

Proteins were electrophoresed through either 6 or 12% acrylamide gels at 100V, blotted onto a polyvinyl membrane at 400mA for 1 or 2 hours depending on whether one or two

gels were being blotted. PVDF membranes were removed to 1% MeOH to fix proteins to the membrane, rinsed in distilled water and transferred to Blocking Solution (BS) for 30 minutes at room temperature. Membrane was washed from the initial BS with three 5-minute washes in BS. Primary antibody was incubated with membranes for 1 hour at RT to overnight at 4°C depending upon the antigen being detected. Non-specific primary Ab was washed off the membrane as described above and secondary antibody applied always as a 1:1000 dilution. Secondary antibody (Horse-raddish peroxidase (HRP)-conjugated anti-(primary species) IgG) was incubated with the membrane for at least 1 hour at RT, washed off as described and the blot was developed by incubation with 0.01% 4-chloro-1-naphthol solution in Tris-HCl (pH 7.5) with hydrogen peroxide as substrate for HRP.

2.18 Zymography

Platelet protein samples were separated by size by SDS PAGE as described above, however PAGE gels contained 1% gelatine. Gels were washed (2.5% [v/v] Triton X-100) and incubated in digestion buffer (200 mM NaCl, 50mM Tris, 5 mM CaCl₂, 1.0 mM ZnCl₂, 0.02% [v/v] Brij-35, pH 7.6; all chemicals from Sigma) for 18 h at 37°C. Gels were then stained in staining solution (0.5% [w/v] Coomassie blue R250 in 30% [v/v] methanol/10% [v/v] glacial acetic acid in H₂O for 3 h at 23°C, and destained (staining solution without Coomassie blue) to reveal discrete regions of gelatin degradation by gelatinases. A well-characterised sample of human amniotic fluid collected at term during active labour was used as a positive control, which clearly demonstrated the latent and active forms of MMP-2 (72 and 66 kDa, respectively) and

the latent form of MMP-9 (92 kDa), and the minor bands the dimeric form of MMP-9 (approximately 210 kDa) and the lipocalin-MMP-9 complex (125 kDa) (Riley et al., 2001; Riley et al., 2004). Platelets are known to contain both MMP-2 and MMP-9 (Fernandez-Patron *et al.*, 1999).

2.19 Anthrone assay of platelet glycogen content.

Glycogen content of platelets was estimated using the method of Seifter *et al* (Seifter *et al.*, 1950). Platelets (1ml PRP) were pelleted by centrifugation at 13.5K rpm for 1 minute. After removal of the supernatant the pellet was resuspended in 500µl 30% KOH and placed immediately at 100°C for 20 minutes, this step lyses platelets and destroys glycolytic enzymes that would otherwise begin catabolism of glycogen. The boiled solution was then diluted with 1ml of 100% EtOH in order to precipitate glycogen and other glycoproteins which were centrifuged from their supernatant for 15 minutes at 4000 x g. The supernatant was removed and the resultant white pellet was dissolved in 500µl of H₂O. Anthrone (1ml of a freshly prepared 0.2% solution in H₂SO₄) was added to the dissolved glycogen and boiled for 10 minutes. A colour change from yellow to blue / black occurs due to the reaction of anthrone with glucose monomers liberated by acid hydrolysis from glycopeptides and glycogen. Absorbance of boiled solutions was performed was read at 625nm using anthrone solution as a zero baseline reading and a serial dilution of glucose as a standard curve (see appendix fig. 8.2). Since inception of the assay many groups working on a diverse range of cell types have used it extensively for the estimation of cellular glycogen (Carroll et al., 1956; Murphy and Gardner, 1971; Chun and Yin, 1998; Muriel and Deheza, 2003).

2.20 Isolation and culture of murine peritoneal macrophages.

Mice were sacrificed by dislocation of the cervical vertebrae. The peritoneal cavity was injected through a 19-gauge needle with 5ml of ice cold sterile PBS. Whilst the needle remained within the peritoneum the abdomen was gently squeezed several times in order to mix the PBS around the cavity. As much of the injected solution was then aspirated from the peritoneum and then transferred to 15ml Falcon tubes and the cell suspension was held on ice until washing. Time of transfer from the animal housing facility to the laboratory laminar flow cabinets was within 5 minutes. Cell suspensions were washed by initially centrifuging cells at 4°C at 300 x g for 5 minutes. The cell pellet was then suspended in DMEM to a final concentration of 1×10^6 cells/ml. Cells were then seeded onto 24 well culture dishes (1ml of cell suspension / well) and allowed to settle for 2 hours at 37°C. Non adherent cells were washed from the dish with three rinses of pre-warmed DMEM. The remaining adherent cells are enriched in peritoneal macrophages. This protocol is used extensively within and without this laboratory for the enrichment of murine peritoneal macrophages.

2.21 Phagocytosis of platelets by mouse peritoneal macrophages.

To demonstrate that dead platelets could be recognised and phagocytosed by macrophages peritoneal cells collected as described above were incubated with cell tracker green labelled platelets (suspended in DMEM) that had been aged for 6 days at 37°C (a time point that was consistently associated with >90% platelet death). Incubation was carried out at room temperature for 30 minutes. Cells were then stained

with Hoechst 33342 to label nuclei, and lysotracker red (100nM). These dyes accumulate quickly within their target organelles (within 5 minutes at RT) and allow the discrimination of nuclear morphology (Hoechst) and phagolysosome localisation (LTR). Platelets alone did not uptake the dyes to amounts remotely equivalent to the macrophages and remained non-fluorescent in their respective channels (blue and red, respectively). Thus, co-localisation of the green platelets with equivalently sized LTR^{+VE} structures within the macrophages confirmed internalisation of platelets. Fresh, viable platelets were used as controls and examined for their incorporation into lysosomal compartment of macrophages. No phagocytic events were recorded with fresh platelets indicating that macrophages relied on the development upon platelets of a recognition signal that promoted their association and phagocytosis by macrophages.

2.22 Statistics.

The Paired Students T-Test was used to establish significant differences between means with P values below 0.05 being deemed significant. For multiple parameter studies such as time courses and comparisons between more than two means Analysis of Variance (ANOVA) test was used. Means are always expressed with standard errors of the mean (+/-SEM).

3 THE PHENOTYPE OF DEAD PLATELETS.

3.1 Abstract.

Platelet death and its consequences for haemostasis are poorly understood. In an attempt to understand events associated with platelet death a number of novel vital stains were identified as useful tools for the assessment of platelet viability. Predictably, platelets incubated over time at 37°C lost the ability to aggregate, activate integrins and degranulate in response to agonists. Electron microscopy revealed a necrotic-like endpoint for platelets stored *in vitro*. Platelets lost the ability to accumulate Calcein-AM and the cardiolipin-specific stain 10-*N*-nonyl acridine orange but concomitantly became able to bind the styryl lipophilic dye FM4-64. All stains appeared to discriminate a platelet subpopulation that displayed morphological characteristics of cell death such as cytoplasmic condensation and reduced forward and side scatter by flow cytometry. Platelet death appeared to be analogous to the platelet storage lesion and the phenotype of platelets within thrombi *in vivo*.

3.2 Results.

3.2.1 Platelets lose functional responsiveness to agonists during *in vitro* storage at 37°C.

It is accepted widely that platelet storage and senescence are associated with loss of function. To confirm that platelets were losing functional responsiveness during *in vitro* storage they were subjected to tests of both degranulation and integrin activation as assessed by increased surface expression of CD62P and fibrinogen binding (fig 3.1A and B, respectively) in response to TRAP. Both assays indicated a progressive loss of function during storage *in vitro*; CD62P expression in response to 20 μ M TRAP was reduced by approximately 50% at 48 hours of storage, whereas fibrinogen binding was reduced by over 80% by the same time ($n=3$, $P<0.01$ for both assays). Furthermore, aggregation in response to calcium ionophore, A23187 (10 μ M) (fig. 3.2) indicated a progressive and significant reduction in the platelets' ability to aggregate during storage with complete loss of aggregability achieved by 96 hours of incubation ($P<0.01$).

3.2.2 Annexin-V binds a sub-population of *in vitro*-aged platelets.

Annexin-V has been used to identify dead cells, to establish platelet death *in vitro* (Brown *et al.*, 2000) and to identify senescent platelets *in vivo* (Pereira *et al.*, 2002). In this work fresh platelets did not bind annexin-V whereas a sub-population of platelets aged *in vitro* for 24 hours did ($P<0.01$) (fig. 3.3), however there was no difference in results for platelets incubated in PRP and those washed and incubated in calcium-free HBSS ($P=0.336$; $n=4$ different donors).

3.2.3 Ultrastructural changes.

Platelets freshly isolated from human donors were discoid, contained discrete granules, glycogen particles, uniformly electron-dense cytoplasmic material and mitochondria (fig. 4A). Overnight incubation at 37°C led to the appearance of a sub population of platelets that displayed varying degrees of plasma membrane integrity (from fully intact to grossly degenerate). Some granules within these platelets were apparently retained in an intact form without signs of degranulation / plasma membrane fusion, whereas others appeared to have fused with one and other. Cytoplasm was collapsed into clumps. These platelets were phenotypically similar to the 'lysed and ballooned' platelets observed previously in stored platelet concentrates (Solberg *et al.*, 1986) and within mural thrombi formed *in vivo* (fig. 3.4.C) (Jorgensen *et al.*, 1967; Davies *et al.*, 1975) or as a result of *in vitro* aging (Brown *et al.*, 2000). The remaining platelets retained their resting morphology and had no signs of activation (fig 3.4.B).

3.2.4 10-Nonyl acridine orange (NAO) fails to accumulate in a sub-population of *in vitro* aged platelets.

NAO specifically associates with cardiolipin and was used to assess the presence in platelets of mitochondria independently of their membrane potential. The dye at 500pM accumulated within fresh platelets within 10 minutes of incubation at 37°C, whereas platelets incubated for 24 hours contained a sub-population that showed morphological signs of cell death (spherical rather than discoid shape, condensed cytoplasm and large vacuolated areas) that were unable to accumulate the dye (fig. 3.5.A). Cytometric

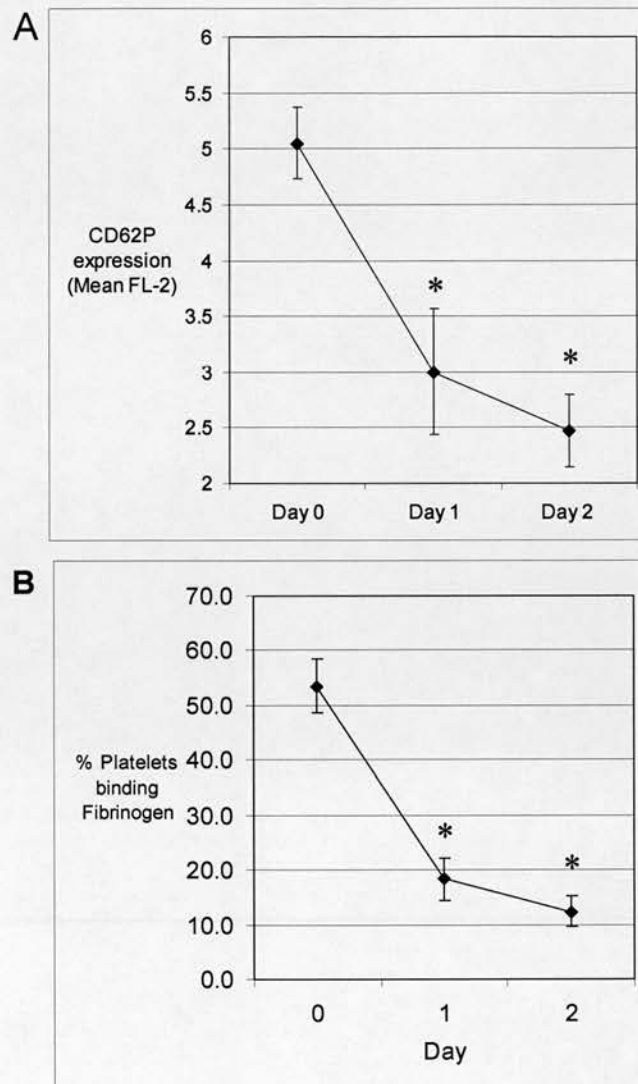


Figure 3.1. Platelets lose the ability to express CD62P and bind Fibrinogen in response to TRAP during *in vitro* storage at 37°C.

Freshly isolated or *in vitro*-aged platelets were activated with 20 μ M TRAP in the presence of Alexa⁴⁸⁸-conjugated fibrinogen, fixed and examined for surface expression of CD62P (A) and fibrinogen binding (B). A significant decrease in both parameters is observed by 24 hours of storage and further decreases by 48 hours. $n=3$ different donors; \pm -SEM; * $P<0.01$ from day 0.

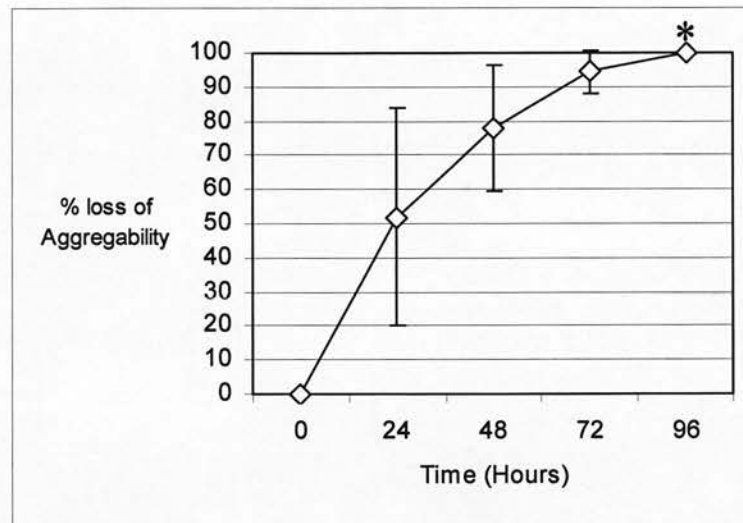


Figure 3.2. Platelets lose the ability to aggregate in response to calcium ionophore, A23187 during *in vitro* storage at 37°C.

Freshly isolated or *in vitro*-aged platelets were activated with 10 μ M A23187 in the presence of 2mM calcium chloride and allowed to aggregate for 5 minutes. $n=3$; \pm SEM; * $P<0.01$.

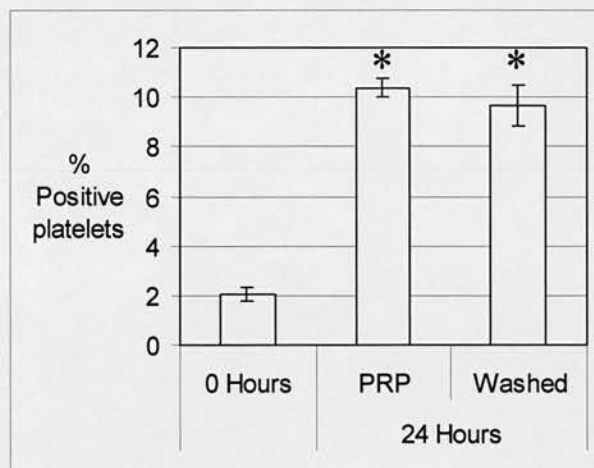


Figure 3.3. Platelets become able to bind Annexin-V during *in vitro* storage at 37°C.

Platelets were incubated at 37°C for 24 hours as PRP or washed and suspended in calcium-free HBSS. Platelets were stained with annexin-V in HBSS containing 2mM CaCl_2 in an iced water bath for 5 minutes and analysed by flow cytometry for positive events using unstained platelets as an autofluorescent control. $n=4$; \pm SEM; * $P<0.01$ from Time 0.

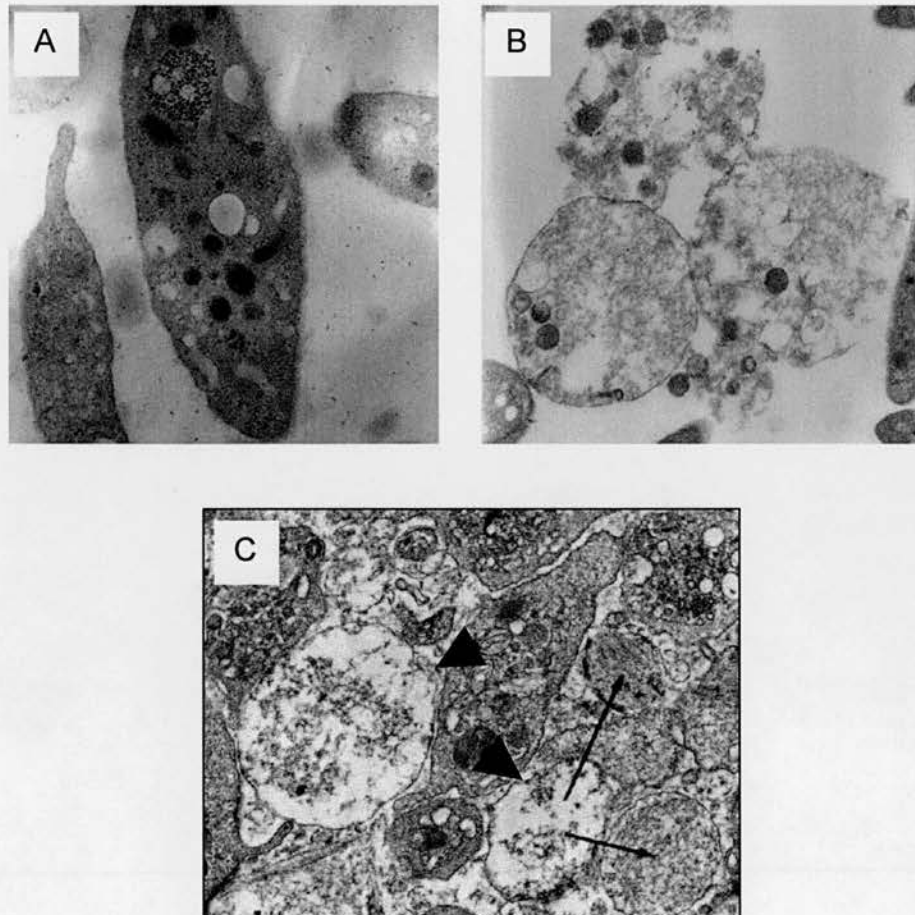


Figure 3.4. The death of platelets is associated with a necrotic morphology.

Platelets were freshly isolated (A) or incubated as platelet rich plasma for 24 at 37°C (B), fixed with 3% gluteraldehyde and processed for TEM. A micrograph from Jorgensen *et al.*, (Jorgensen *et al.*, 1967) illustrates the similarity between *in vitro* aged platelets and platelets within an experimentally induced porcine arterial thrombus (arrowheads, C). Original magnification in A and B x17,000. Micrographs are representative of many platelets from several donors.

analysis of platelets revealed that 3.8 \pm 1.7% of freshly isolated platelets failed to accumulate the dye. During storage the NAO^{-VE} subpopulation became increasingly common and rose to 15.2 \pm 3.1% at 24 hours; $n=3$ different donors; $p<0.01$ (fig. 3.5.B.i). Back-gating on the NAO^{-VE} population revealed that these platelet events had reduced forward and side scatter compared to NAO^{+VE} events (fig 3.5.B.ii).

3.2.5 Dead platelets fail to accumulate calcein-AM but bind FM4-64.

Freshly isolated platelets incubated in the presence of 10nM (for cytometry) or 100nM (for microscopy) calcein-AM became fluorescent within 10 minutes (fig. 3.6.A). Washed platelets and platelets in diluted PRP accumulated calcein to a similar level as un-manipulated platelets. By microscopy it was clear that calcein was able to accumulate in the platelets' cytoplasm but also to discrete (but as yet unidentified) granules (fig. 3.6A). Incubation of platelets as cPRP, diluted cPRP (dcPRP) or washed and suspended in calcium-free HBSS at 37°C led to the appearance of a subpopulation of platelets that could no longer accumulate calcein. There was no change in the ability of the main platelet population to accumulate the dye (fig. 3.7) indicating that platelets' ability to accumulate the dye is lost abruptly rather than gradually. Morphological examination by microscopy indicated that these calcein^{-VE} platelets shared characteristics with the lysed and balloon platelets seen at the TEM level. The calcein^{-VE} platelets were spherical and had collapsed cytoplasm with residual material and granules associated with the periphery of their plasma membrane.

As part of a separate study examining the role of the lysosomes in platelet biology it was noted that although freshly isolated platelets poorly accumulated the yeast vacuole marker FM4-64 the calcein^{-VE} platelets were ten-fold more brightly stained with the dye. The dye appeared to be inserted into the plasma membrane of dead platelets with some staining associated also with residual cytoplasmic material (fig. 3.6). Bivariate analysis of aged platelets indicated that the calcein^{-VE} platelets were FM4-64^{+VE} and that no other sub-population existed (fig. 3.7.A).

Activation of fresh platelets with 20μM ADP or 50ng/ml convulxin in the absence of stirring indicated that the platelets' activation status did not affect the accumulation into platelets of calcein nor did it allow the insertion of FM4-64 into platelets (fig. 3.8.A, B and C).

3.2.6 Calcein-VE / FM4-64+VE platelets fail to bind fibrinogen or exhibit a calcium response when stimulated with convulxin.

The morphological data collected at the light and electron microscopic level indicated that a population of dead platelets arose during *in vitro* storage at 37°C. Although there was correlation between this morphology and changes to the platelets' ability to accumulate calcein and NAO or allow the insertion of FM4-64 into their plasma membrane, it was necessary to demonstrate loss of function in these platelets. To establish that these platelets could no longer activate integrins nor evoke calcium response they were stimulated with convulxin after staining with FM4-64.

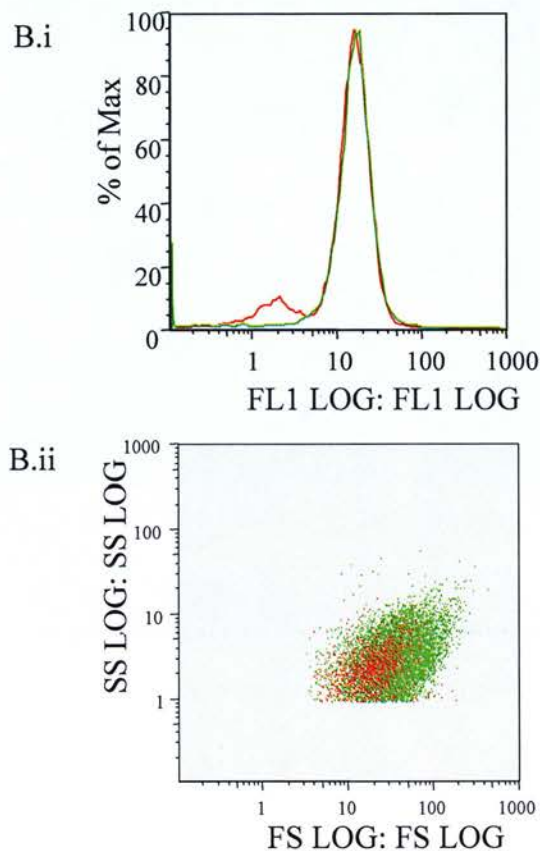
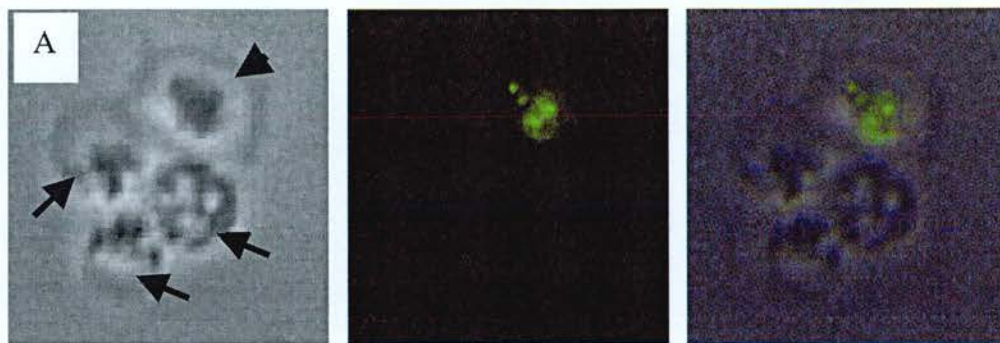


Figure 3.5. Platelets displaying signs of death fail to accumulate NAO.

Platelets were aged *in vitro* at 37°C for 24 hours and stained with 500pM nonyl-acridine orange for 10 minutes. Incubation of platelets with NAO defines two populations that either do or don't accumulate the dye. (A) Micrographs depict 4 platelets aged *in vitro* for 24 hours; only one platelet has accumulated NAO (arrowhead), three platelets displaying features of cell death (loss of resting shape, condensed cytoplasm and vacuolisation) failed to accumulate the dye (arrows). (B.i) Cytometric analysis of NAO-stained fresh (green) or *in vitro* aged (red) platelets is shown. (B.ii) Back-gating on NAO-negative platelets reveals they have decreased forward scatter relative to NAO positive platelets. Micrographs and cytometry data are representative of stored platelets from many different donors (>30).

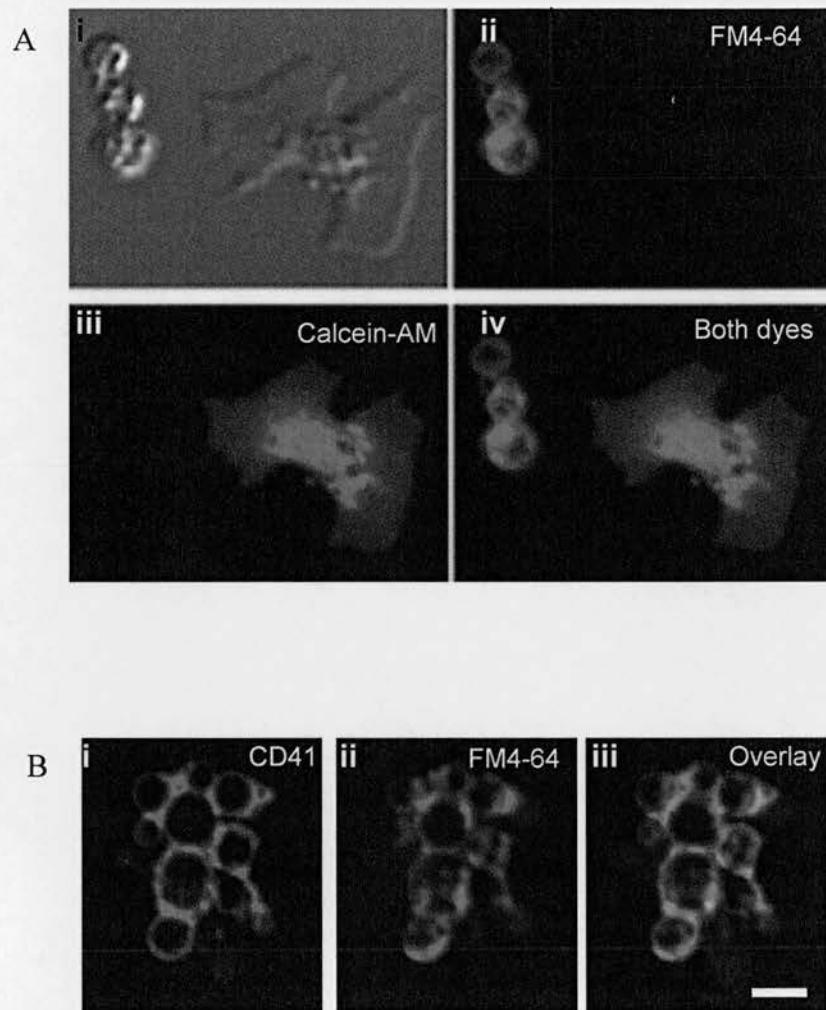


Figure 3.6. Platelets displaying signs of death are FM4-64 positive and calcein negative.

Platelets were incubated for 24 hours at 37°C, stained with 100nM calcein-AM and 10 μ M FM4-64 for 10 minutes and allowed to spread on glass for 5 minutes at room temperature. Viable platelets were calcein positive but FM4-64 negative whereas dead platelets showed the opposite staining pattern (A). Confirmation that dead cells were indeed platelets was sought with anti-CD41 staining (B). Bar = 3 μ m. Micrographs are representative of platelets from many different donors.

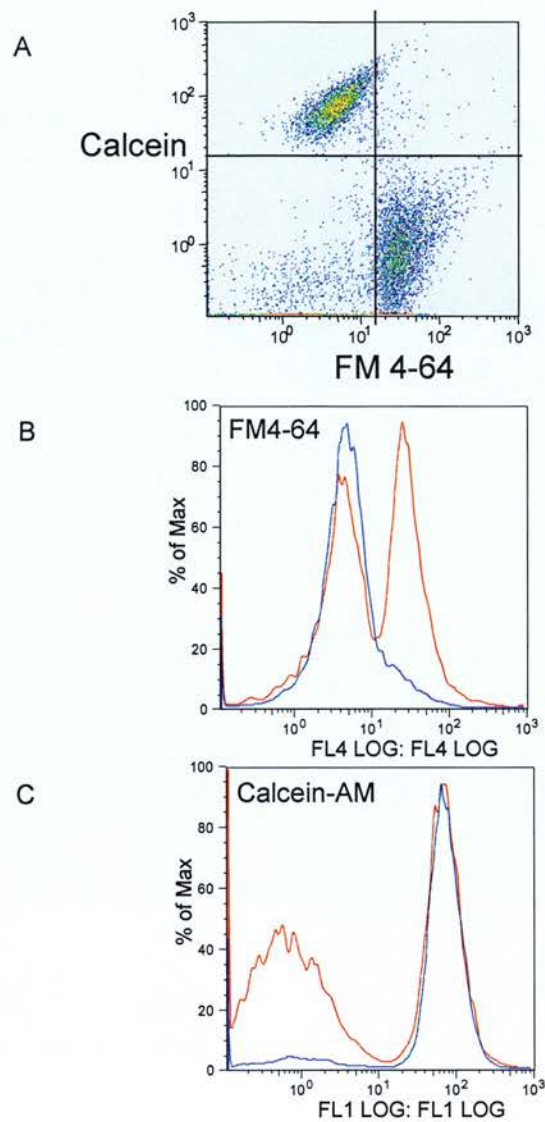


Figure 3.7. Subpopulations of platelets can be identified by cytometry either with Calcein-AM or FM4-64.

Platelets were aged for 72 hours and stained with both 100nM Calcein-AM and 5 μ M FM4-64. Bivariate analysis establishes that calcein-AM negative platelets are FM4-64 positive (A), thus the dyes can be used interchangeably. Histograms of fresh and stored platelets (blue and red lines, respectively) illustrate the fluorescence shift associated with platelet storage for FM4-64 (B), a positive shift to the right; for calcein-AM (C) a negative shift to the left.

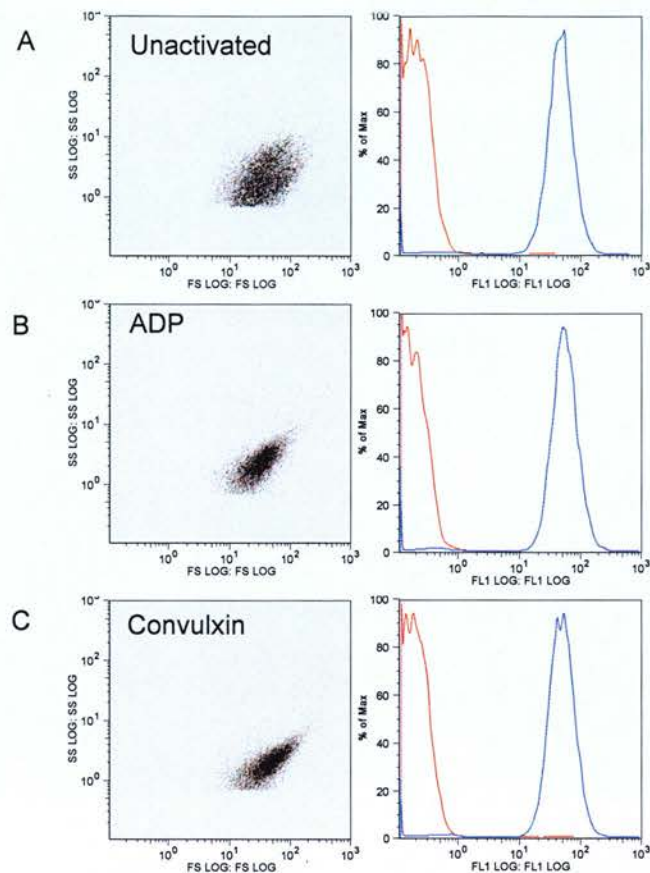


Figure 3.8. Activation does not affect platelets ability to accumulate Calcein-AM.

Platelets were activated in the presence of 2mM CaCl for 10 minutes with no agonist (A); 20 μ M ADP (B) or 50ng/ml convulxin (C) and stained for a further 10 minutes with 10nM calcein-AM. Autofluorescence was set within the first log order of brightness with non-activated, unstained platelets. Activation can be confirmed as a change in forward and side scatter from a resting-state dot plot (A) to an activated 'comet-tailed' dot plot scatter (B and C). Data are representative of data from two different donors.

Activation of platelets with convulxin after being incubated for 24 hours indicated that FM4-64^{+VE} platelets failed to activate $\alpha_{IIb}\beta_3$ integrin complex (fig. 3.9) and failed also to produce a calcium signal (fig. 3.10) as evidenced by their lack of ability to bind labelled fibrinogen and exhibit increased Fluo-3 fluorescence signal, respectively. Similar data were obtained with thrombin and ADP indicating that the inability to respond to agonists was not unique to a single receptor or signalling pathway (data not shown).

3.2.7 Platelets die less quickly at ambient temperature than when at 37°C.

Platelets stored for subsequent transfusion are kept at 20°C. This temperature is a compromise due to the fact that platelets stored at lower temperatures are cleared rapidly from circulation upon transfusion, a process thought to be mediated by CR3 on hepatic macrophages (Hoffmeister et al., 2003). Although there is much interest in improving platelet storage conditions few reports exist as to the retention of viability of platelets at various storage temperatures.

Therefore it was of fundamental interest to establish the rate of death for platelets stored as PRP at both 37°C and ambient temperature (estimated to range from 18 to 22°C). Bivariate analysis of platelets stained with calcein-AM and FM4-64 indicated that platelets died at the significantly different rates of $13.5 \pm 0.9\%$ platelets.day⁻¹ and $6.0 \pm 1.5\%$ platelets.day⁻¹ at 37°C and ambient temperature, respectively ($n=3$ different donors; $P < 0.01$) (fig. 3.11).

3.2.8 Dead platelets are phagocytosed by mouse peritoneal cells.

The ultimate fate for many dead cells lies within the phagolysosome of professional macrophages and semi-professional phagocytes. This process of effete platelet destruction has been demonstrated *in vitro* for platelets and is confirmed in this work. Platelets stained with Cell tracker green (CTG) and aged for 6 days (a time-point that is associated with >90% platelet death) were introduced to mouse peritoneal lavage cells that are routinely used in this lab for the isolation of macrophages. Although the crude cell population contains monocytes, neutrophils and epithelial cells it is found that only peritoneal resident macrophages are capable of ingesting apoptotic neutrophils. Therefore it was assumed that observation of any phagocytic events was evidence of an interaction between macrophages and dead platelets. Confirmation of internalisation was achieved using lysotracker red that accumulated within macrophage lysosomes but did not associate with platelets. Examination of lysotracker red-stained cells after 30 minutes indicated the presence of green platelets within red lysosomes compartment (fig. 3.12), confirming the observations made by others (Brown et al., 2000; Clarke et al., 2003) that dead platelets produced *in vitro* are a target for phagocytosis.

3.2.9 Microvesiculating platelets are calcein-VE and FM4-64+VE.

It is likely that microvesicles shed from platelets are not viable cellular entities. It was therefore reasoned that microvesiculation could be identified using calcein-AM and FM4-64. To confirm this hypothesis platelets that were allowed to spread on glass were then treated with either DMSO or 10 μ M A23187 to induce vesiculation and subsequently stained with calcein and FM4-64 (fig. 3.13). Control platelets (DMSO

treated) accumulated calcein but very little FM4-64, which appeared to accumulate within the central, granular region of the platelet. Platelets treated with A23187 to induce microvesiculation were unable to subsequently accumulate calcein but did allow the insertion of FM4-64 into their plasma membranes and developing microvesicles.

3.2.10 Calcein and FM4-64 differentiate “medulla” and “cortex” regions of large platelet aggregates.

Although the activation of fresh platelets in the absence of agitation did not lead to changes in their ability to accumulate calcein or FM4-64, the results obtained with vesiculating platelets led to the hypothesis that platelets within aggregates (and thus within an environment of high agonist concentration) would be vesiculating and that the core of an aggregate is essentially dying. To explore this hypothesis platelets were aggregated in response to collagen or TRAP within an aggregometer in the presence of 2mM CaCl₂. Platelet aggregates were then incubated with calcein-AM and FM4-64 for 20 minutes and examined by fluorescence microscopy.

It was clear that larger aggregates of platelets contained a “medulla” region of FM4-64 positive that was devoid of calcein accumulation, whereas the “cortex” was populated by platelets that retained the ability to accumulate calcein but did not bind FM4-64 (fig. 3.14). Phase microscopy revealed also that the region of FM4-64^{+VE} platelets coincided with the deposition of fibrillar material presumed to be fibrin.

3.2.11 Platelet aggregation is associated with platelet degeneration.

Freshly isolated platelets were aggregated in response to fibrillar collagen and allowed to settle for 1 hour at 37°C. The aggregates were then fixed and prepared for electron microscopy. Examination of sections revealed the presence of a complex array of platelet and 'post-platelet' morphologies (fig. 3.15). Although many intact platelets in various stages of activation could be seen there were also many that were devoid of granules, containing dilute cytoplasm with misshapen peripheral membranes that often contained gaps. Large areas of the sections were populated by microvesicles of varying electron density, size and granularity. Furthermore, there were extensive areas of gel-like material of intermediate electron density that was unlike grouped platelets or vesicles. The material existed outside other structures that had limiting membranes and appeared to be confluent, with no recognisable cellular organisation of its own.

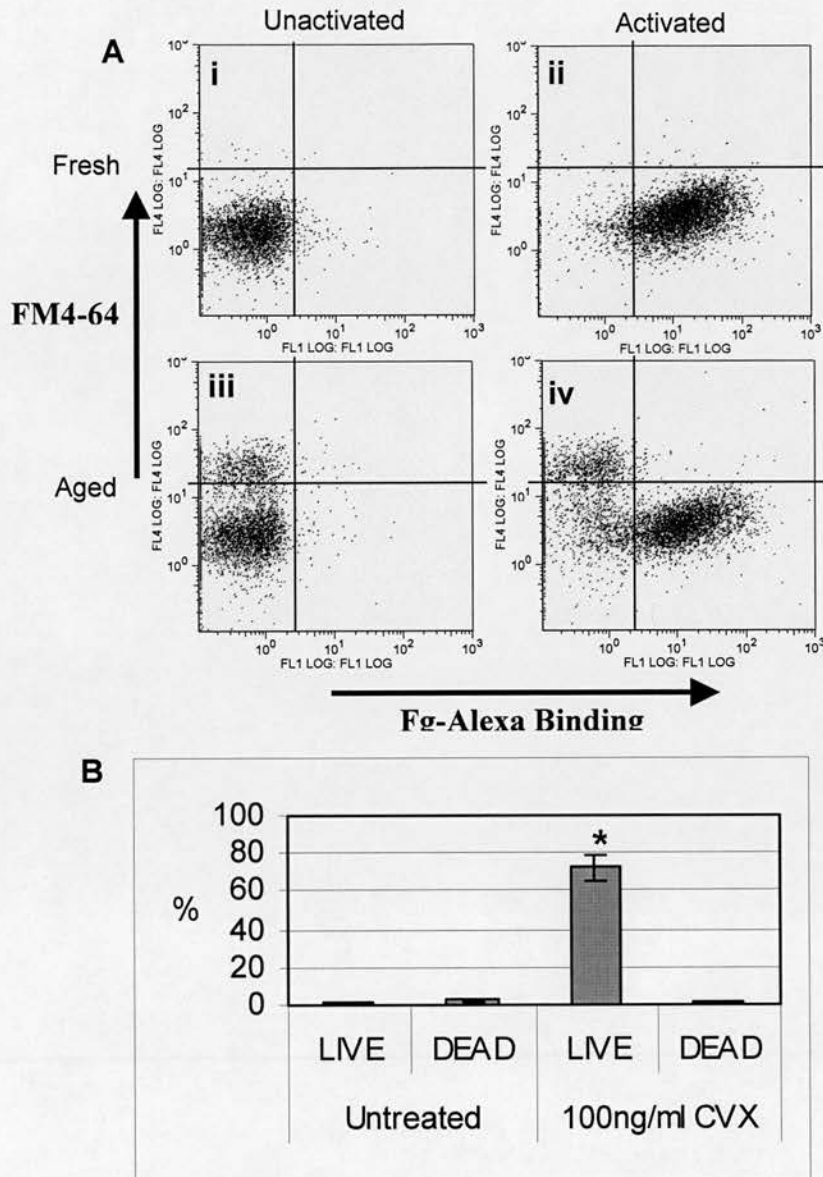


Figure 3.9. Dead platelets are unable to bind fibrinogen in response to convulxin.

Washed platelets were activated with 100ng/ml convulxin in the presence of 20 μ g/ml Alexa⁴⁸⁸-conjugated fibrinogen whilst fresh or after incubation overnight in calcium-free HBSS. Platelets were then stained with FM4-64 to differentiate live from dead and analysed by flow cytometry for fibrinogen binding. Fresh platelets (Ai and ii); aged platelets (Aiii and iv). Non activated platelets (Ai and iii); activated platelets (Aii and iv). Fibrinogen positive platelets are gated in the right quadrants and dead platelets are gated in the upper quadrants. Cytometry plots are representative of data from 4 different donors (A). Quantified data from 4 donors (B); +/-SEM; *P<0.01.

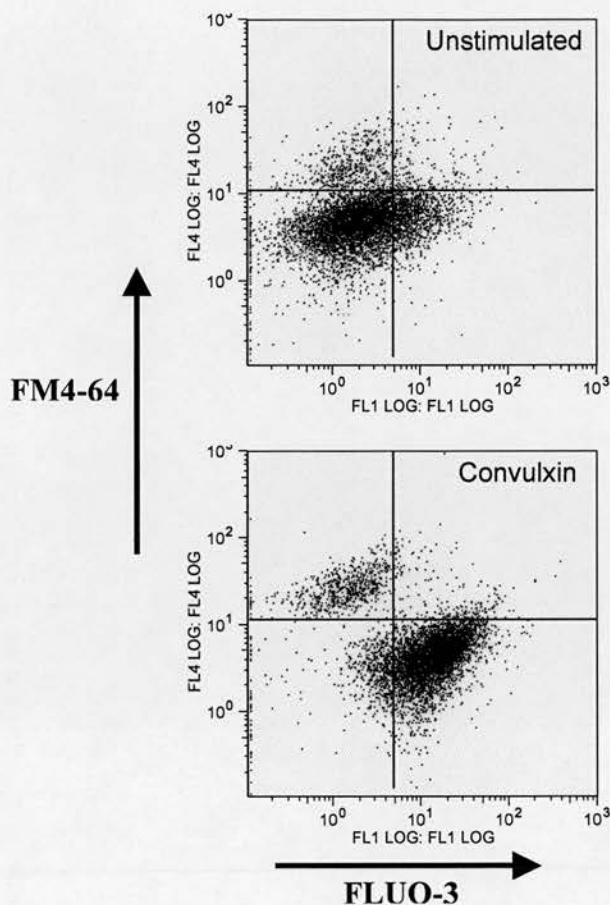


Figure 3.10. FM4-64+VE platelets do not give a calcium signal in response to convulxin.

Platelets were incubated for 24 hours, stained with 5 μ M FM4-64 for 10 minutes and loaded with 1 μ M Fluo-3 for 30 minutes. Platelets were then stimulated with 100ng/ml convulxin in the presence of 2mM CaCl₂ or left in their resting state. Activation was allowed to proceed for 1 minute prior to flow cytometric analysis of green fluorescence. Cytometry plots are representative of results from 2 different donors.

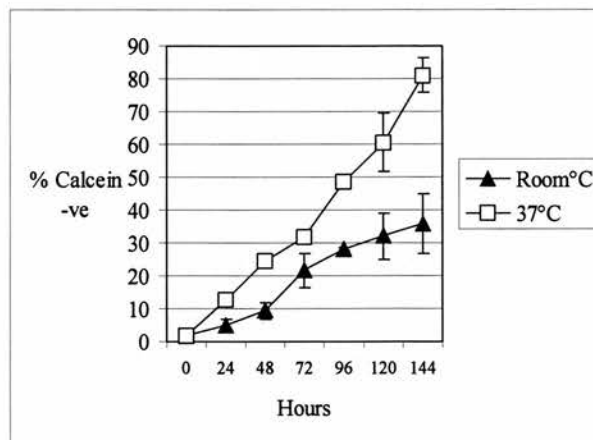


Figure 3.11. Platelet death is slower at ambient temperature.
Platelets were stored as PRP supplemented with 2mM MgSO_4 at either 37°C or ambient temperature for six days. Staining platelets with FM4-64 assessed death. Only CD41 positive events were collected and FM-4-64 positive events were deemed to represent dead platelets. $n = 6$; +/-SEM.

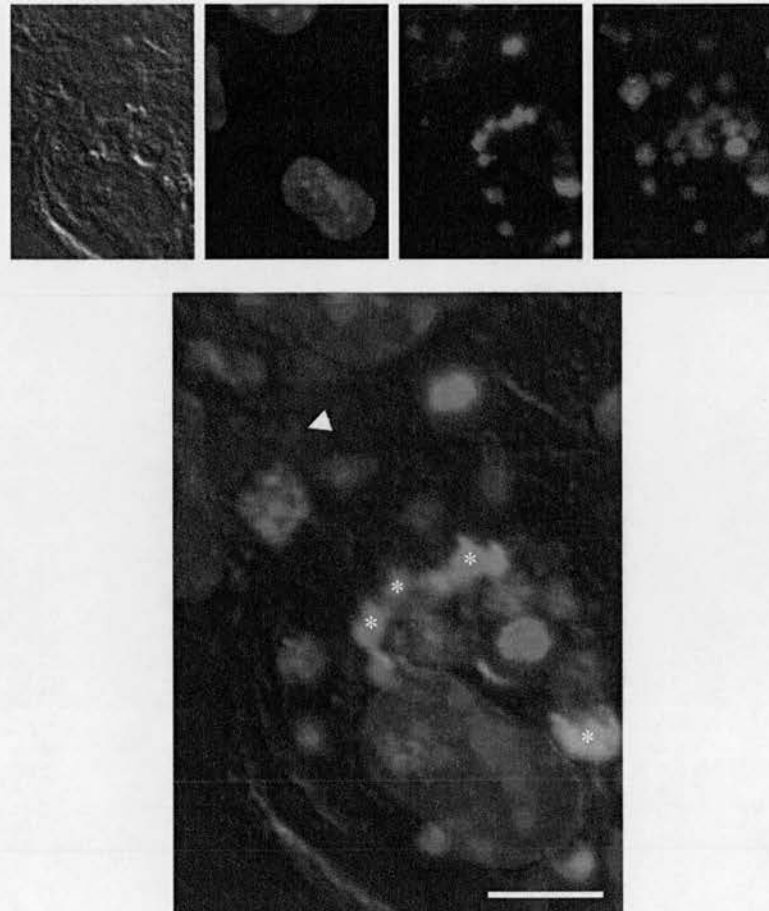


Figure 3.12. Macrophages phagocytose dead platelets.

Cell tracker green (CTG)-labelled platelets were incubated at 37°C as cPRP for 6 days (a timepoint where >90% have lost viability as assessed by their inability to accumulate calcein-AM). Platelets were washed from their incubation fluid and resuspended in DMEM containing 10 % foetal calf serum and incubated in suspension with murine peritoneal cells for 30 minutes at ambient temperature on a Rollimixer. Nuclei and lysosomes were then stained with Hoechst 33342 and lysotracker red, respectively. Phagolysosomes (arrowhead) and phagolysosomes containing CTG^{VE} platelets (asterisks) were readily observed. Platelets outwith phagolysosomes do not colocalise with LTR (arrowhead). Bar = 10µm. Micrographs are representative of data collected from two different donors.

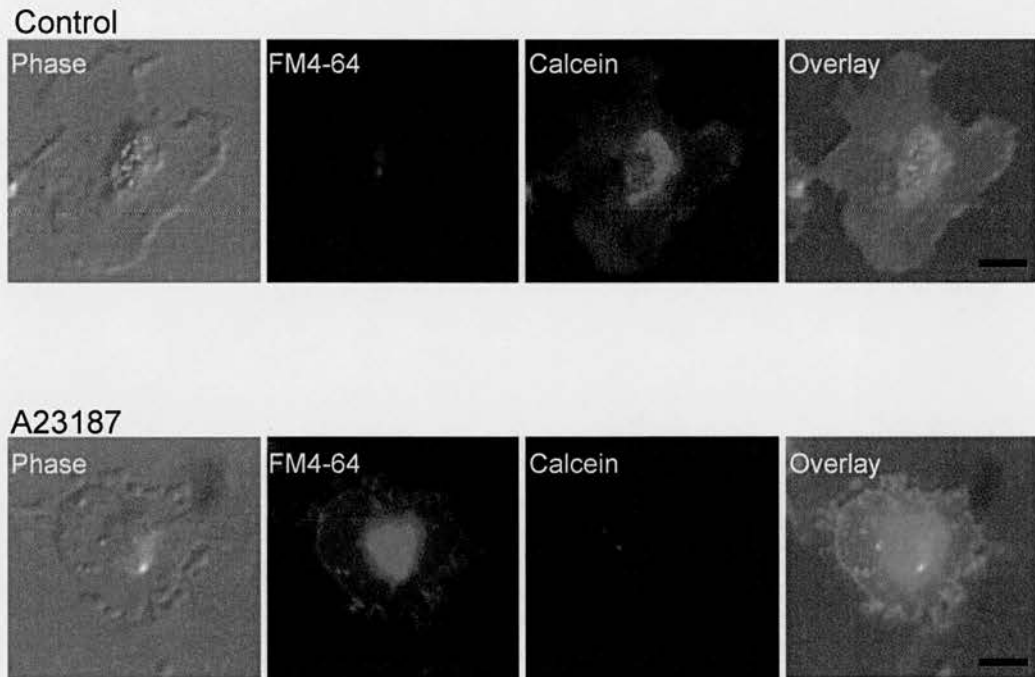


Fig 3.13. Microvesiculating platelets are calcein^{-VE} and FM4-64^{+VE}.

Platelets were allowed to spread for 20 minutes on glass and then treated for 5 minutes with either 0.01% DMSO (control) or 10 μ M A23187. Platelets were then stained with 100nM Calcein-AM and 5 μ M FM4-64. Control platelets accumulated calcein and failed to allow FM4-64 insertion into their plasma membrane, whereas A23187-treated platelets were unable to accumulate calcein but did allow FM4-64 insertion into both developing vesicles and the plasma membrane. Bar = 5 μ m. Images are representative of data collected from several donors.

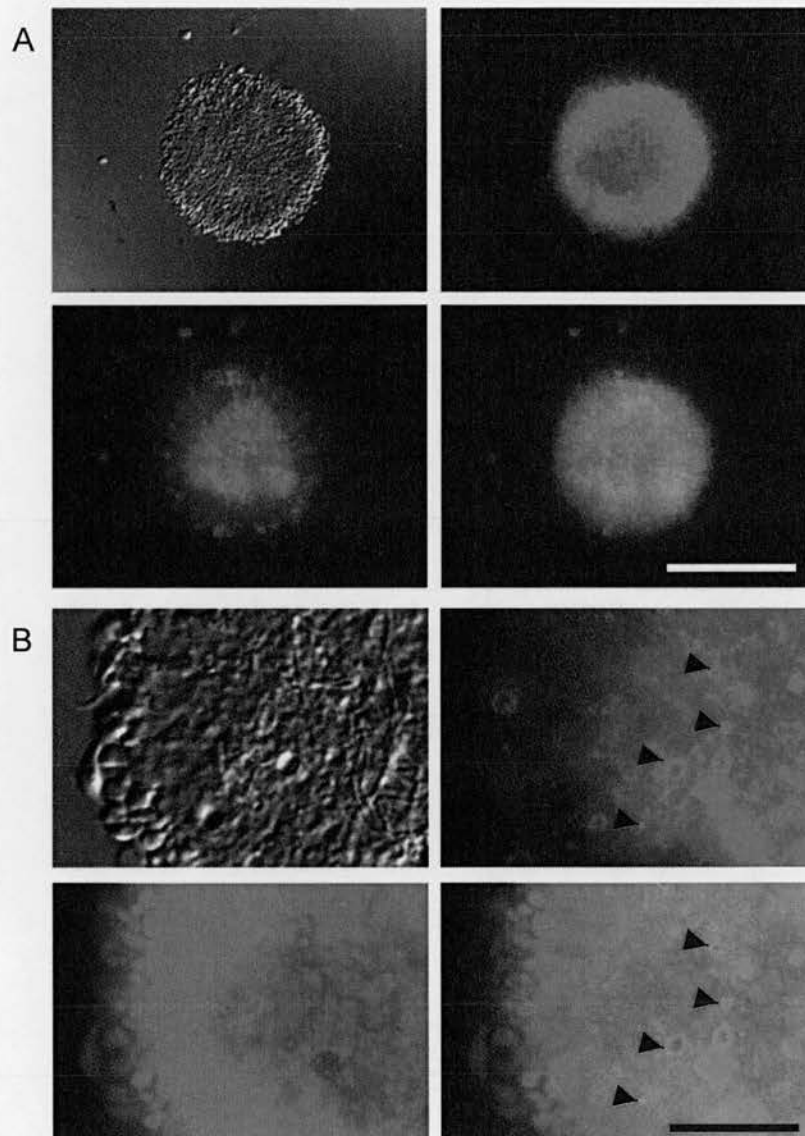


Fig 3.14. Calcein and FM4-64 differentiate a cortex and medulla within larger platelet aggregates.

Platelets were aggregated with 1 μ g/ml collagen for 5 minutes and stained with 100nM calcein-AM (green) and 5 μ M FM4-64 (red) for a further 10 minutes and viewed by epifluorescence microscopy. Aggregates of various sizes consistently showed strict organisation of FM4-64^{+VE} / Calcein-AM^{+VE} platelets to their external surface and FM4-64^{+VE} / Calcein^{low} within their medulla. The apparent death of platelets appears to involve the production of small vesicles that bind FM4-64 but fail to accumulate calcein-AM (arrowheads, B.iv). A fibrillar material (presumed to be fibrin) can be seen associated with areas of apparent platelet death (arrowhead B.i). Bar = 50 μ m (A); 10 μ m (B). Images are representative of data collected from several donors.

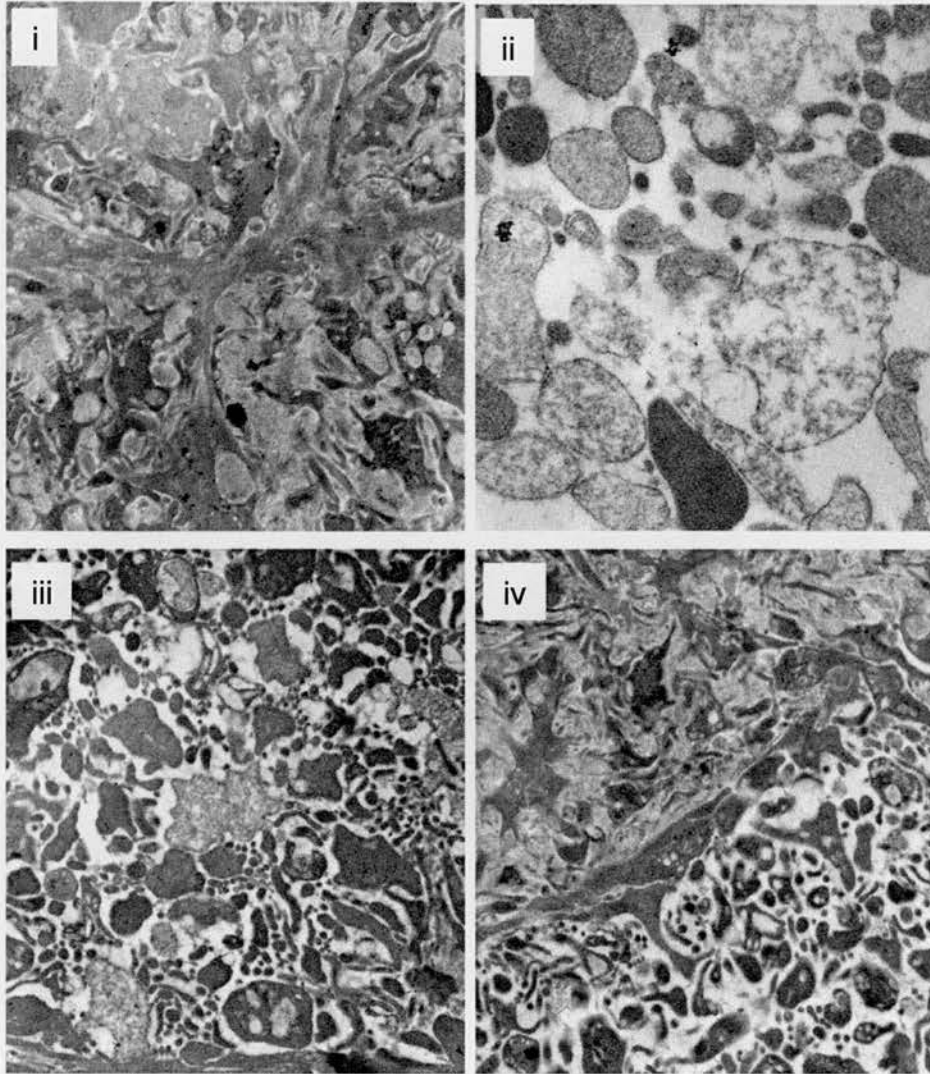


Fig 3.15. TEMs of platelets within collagen induced aggregates 1 hour after formation.

Platelets were aggregated with 1 μ g/ml collagen for 5 minutes and fixed with 1.5% glutaraldehyde and prepared for transmission electron microscopy. A variety of changes appear to have taken place such as the formation of a 'gel' (i and iv), degranulation (ii), necrosis (ii) and microvesiculation (iii and iv). Images are representative of many platelet aggregates from a single donor.

3.3 DISCUSSION.

Despite the clinical and academic importance of platelets there have been very few attempts to phenotype dead or dying platelets, define their program of cell death or relate these changes to alterations in haemostasis. There are clear technical difficulties associated with working on highly refractory cells such as platelets. It is widely understood that without exogenous manipulation of their *ex vivo* environment platelets become activated. The most widely used method achieving this is the chelation of calcium_(ex). However, even when this has been done it is possible still to stimulate platelets to the point of activation using sub-physiological temperatures or agonists such as thrombin and collagen; therefore chelation of calcium does not guarantee the inhibition of platelet activation. A major concern in the current work was whether or not platelet death was the result of activation. Previous work demonstrated that degranulation occurred prior to death but the authors were unable to conclude that degranulation was necessary for the death process (Brown *et al.*, 2000). Due to the similarities between certain types of platelet activation and programmed cell death it was very important to avoid manipulating platelets in order to answer the question of whether or not their death occurs independently of activation. Furthermore, it is unclear what net result platelet activation has upon platelet lifespan *in vitro* or for that matter, *in vivo*.

Loss of functionality is a common feature of platelet storage and was confirmed in this work by examination of aggregation in response to calcium ionophore and integrin

activation and degranulation in response to TRAP. Confirmed also was the development of a sub-population of platelets that could bind annexin-V. It is not clear if annexin-V binding to platelets is a result of lost membrane asymmetry or lost membrane integrity as no dyes were used that could discriminate between the two, however TEM data indicated the presence of platelets that had lost membrane integrity. Deliberate manipulation of membrane integrity with detergents such as saponin followed by assessment of annexin-V binding may help to resolve this question.

The current data are in disagreement with a previous report that incubation of platelets in the absence of plasma leads to accelerated cell death (Brown *et al.*, 2000). The reasons for this are unclear but may reflect a lack of magnesium (a crucial cofactor for ATP) in the plasma-free incubation medium and / or the 5-fold greater amount of CaCl_2 in the annexin-V binding buffer (10mM *versus* 2mM used in the present work) or a combination of factors that promoted platelet PS exposure in response to annexin-V binding buffer.

Previous examination of platelets aged *in vitro* led to the hypothesis that a program of cell death occurs that is independent of caspase activity (Brown *et al.*, 2000). Ultrastructural examination of the platelets aged as platelet rich plasma or as diluted PRP revealed the presence of two conspicuous platelet populations, a resting population and one where resting shape was lost to a spherical morphology with dilute internal cytoplasm that was condensed in some areas. This population showed signs of granule fusion, organelle loss, loss of glycogen and loss of plasma membrane integrity. Apart from the formation of large vacuoles that appeared to be independent of platelet death

(discussed in a later chapter) there was no evidence of a progressive death phenotype over and above a sudden and *catastrophic* loss of cellular integrity. Interestingly the caspase-independent cell death program described previously was not associated with signs of necrosis, despite TEMs showing gaps within the plasma membranes of *in vitro* aged platelets (Brown et al., 2000; Clarke, 2002). The progression of platelets in that model also appeared to involve P-selectin expression, something that was not seen in the current work, thus platelet death can occur independently of P-selectin expression. This raises the possibility that P-selectin expression in the previous model was due to activation and that the phenotype of dying platelets was affected by this event.

The phenotype described in this thesis is not uncommon in the literature, being similar to platelets treated with a combination of thapsigargin and thrombin (Stuart et al., 1995) or the calcium ionophore, A23187 (Gerrard et al., 1974) but it is similar also to the phenotype of platelets that develop platelet storage lesion during *ex vivo* storage. Platelets activated with ionophore or with physiological agonists in the presence of thapsigargin display within seconds sphering, dilution of their cytoplasm and microvesiculation. Thapsigargin is known to increase intracellular calcium concentration due to inhibition of calcium uptake into the dense tubular system (Engelender *et al.*, 1995). In synergy with agonist it has a potent effect on the platelets' plasma membrane lipid asymmetry and allows for the study of annexin-V binding to exposed PS (Stuart et al., 1995; Dachary-Prigent et al., 1995). Also the phenotype is similar to that induced by the calcium ionophore A23187. This platelet agonist is non-physiological but is used widely for the study of platelet biology. The ionophore abolishes divalent cation gradients within cells by binding and transporting cations

across membranes; thus it causes dramatic emptying of calcium stores and a rapid rise in cytoplasmic calcium concentration in platelets leading to activation and, in particular, microvesicle formation with PS exposure (Chang et al., 1993; Dachary-Prigent et al., 1993). Other agonists such as ADP or thrombin produce less dramatic events such as integrin activation and degranulation and are not associated with PS exposure or vesiculation unless in conjunction with collagen (or thrombin). Both the calcium ionophore and thapsigargin based platelet activation strategies are known to induce the formation of microvesicles by platelets, a process that appears to depend upon the influx of extracellular calcium through store-operated calcium channels (SOCCs) (Pasquet *et al.*, 1996) and the activation of calpains (Wolf *et al.*, 1999).

Microvesicle formation during storage of platelets was not observed either at the electron or light microscopic level in this thesis. This may be a reflection of the low calcium environment of platelet storage, which could conceivably prejudice any contribution that an influx of calcium into platelets has on the vesiculation process; however this pre-supposes that calcium stores are depleted during storage and that SOCC has become activated, a possibility examined in Chapter 6.

The phenotype of dead platelets is most similar to descriptions of the platelet storage lesion (Murphy and Gardner, 1969; Solberg et al., 1986) a common but poorly understood consequence of platelet storage *ex vivo* for transfusion purposes. It is likely that PSL and the platelet death observed in this work and by others (Brown et al., 2000; Clarke et al., 2003) are the same. PSL is known to be associated with a toxic increase in intraplatelet lactate (Bode and Miller, 1989; Bertolini et al., 1993) and raises the

possibility that if a catastrophic loss of integrity was witnessed in this thesis it was due to a metabolic dysfunction which led to necrosis, a process that can have many similarities to programmed cell death (Kroemer et al., 1998; Vande Velde et al., 2000).

To avoid the labour intensity associated with electron microscopy a reliable high-throughput methodology for the analysis of dead platelets was sought. During the course of the thesis it was of interest to establish the whereabouts of mitochondria using a dye that accumulated in the organelles independently of their $\Delta\Psi_m$. It was found that 10-*N*-nonyl acridine orange was able to accumulate to small structures within platelets consistent with its ability to associate with cardiolipin, a charged phospholipid found exclusively in mitochondria. NAO accumulated in >95% of freshly isolated platelets but failed to accumulate within a sub-population of *in vitro* aged platelets displaying signs of lesion, suggesting a qualitative change to mitochondria / cardiolipin that occurred at this point which prevented the accumulation of NAO within platelets. Microscopic examination of stored platelets consistently revealed that only those platelets exhibiting lesion failed to accumulate the dye.

This prompted two questions; if NAO was accumulating independently of $\Delta\Psi_m$ what second event caused loss of NAO accumulation and is this second event the cause or consequence of lesion? To examine these questions in detail the mitochondria and changes to them caused by experimental manipulation were examined and form the body of work presented in another chapter.

It was found that the liberation of fluorescent calcein from calcein-AM could be used as an indicator of platelet viability. Freshly isolated platelets readily accumulated the dye in their cytoplasm and more so to discrete granules. Calcein^{+VE} platelets could spread on glass, indicative of viability and responded to agonists such as TRAP and ADP. Platelets accumulated calcein comparably well after 10 minutes under unstirred conditions in the presence of maximal doses of ADP, CVX or TRAP; therefore calcein accumulation occurred independently of single platelets' activation status.

Microscopic and cytometric examination of platelets incubated for 24 hours at 37°C and then 10 minutes in calcein-AM at 37°C revealed that a subpopulation of platelets could no longer accumulate the dye; by microscopy these platelets were morphologically distinct from calcein^{+VE} platelets. Calcein^{-VE} platelets showed signs of PSL such as cytoplasmic condensation and did not spread on glass nor respond to agonists. The calcein^{-VE} platelets had also reduced forward and side scatter characteristics by flow cytometry, another identifier of cell death. It is not clear if calcein-AM fails to accumulate in lesioned platelets due to loss of esterase activity or due to loss of membrane integrity. However, it is likely that these platelets are the same ones that cannot accumulate NAO. This hypothesis was not tested because the molecules have similar fluorescence emission spectra that cannot be differentiated by cytometry. Nonetheless, the rate of platelet lesion over several days *in vitro* was similar whether examined with NAO or calcein. Thus the abilities to accumulate NAO and liberate calcein are lost simultaneously. The observation at the TEM level that plasma membrane integrity is lost in platelets exhibiting other signs of PSL is suggestive that the ability of platelets to accumulate calcein is dependent on them having an intact

plasma membrane. Loss of plasma membrane integrity would allow annexin-V to bind PS on both the endo- and exofacial sides of the plasma membrane (which is why plasma membrane-sensitive dyes are used in conjunction with annexin-V to discriminate between apoptosis and necrosis). Thus it is possible that the lesion / death of platelets detected by annexin-V, NAO and calcein-AM is the result of a loss of plasma membrane integrity leading to swelling of platelets due to influx of solutes.

It was demonstrated that the platelets displaying signs of cell death by morphology could associate with substantially more of the yeast vacuole marker FM4-64 than viable platelets. The reason for the association of this dye with dead platelets is not understood, however similar results are obtained with dead neutrophils (the dye binds only the apoptotic and necrotic cells). When used with viable yeast the dye inserts into the plasma membrane at low temperatures and within 20 minutes of transfer from 4°C to 25 °C endocytosis of the dye and its accumulation at the vacuole can be observed (Vida and Emr, 1995), however this does not appear to occur with fresh platelets. Even with extended periods of time at 37°C, the dye did not associate with the plasma membrane or accumulate within discrete organelles of fresh platelets. This may reflect the dye's usage at a lower concentration in this study than with yeast (5µM compared to 40µM with yeast) but may also suggest fundamental differences between the plasma membranes of yeast and that of platelets / animal cells. Dye insertion into the plasma membrane of dead platelets may reflect the known changes in membrane asymmetry and charge that accompany cell death (Morris et al., 1984; Savill et al., 1989). Dual labelling of platelets indicated that association of FM4-64 with dead platelets' plasma membrane occurs concomitantly with the platelets' inability to accumulate calcein and

that no other platelet populations were defined. Using FM4-64 it was confirmed that FM4-64^{+VE} platelets were unable to respond either by initiating a calcium signal or binding fibrinogen in response to strong agonist such as convulxin. It was demonstrated also that dead but not viable platelets could be internalised by phagocytes. These data are strong arguments in favour of the conclusion that NAO; calcein and FM4-64 are capable of detecting dead platelets. Although there is strong evidence for a necrotic endpoint for platelets *in vitro* it is not possible to determine if the dyes utilised in this study are capable of detecting only necrotic or a mix of both pre and post-necrotic platelets.

It was found that platelets aged for several days at 37°C died more rapidly than those kept at ambient temperatures. The use of vital stains in conjunction with cytometry is a worthwhile means of exploring platelet storage conditions.

Platelet micro-vesicle formation has long been regarded as a process similar to that of apoptotic 'blebbed' body formation, however microvesiculation is effected by calpain rather than caspase activity (Wolf *et al.*, 1999) and there is indirect evidence suggesting that PS exposure associated with platelet vesiculation is distinct from that observed during apoptosis (Williamson *et al.*, 2001). Despite these differences it was probable that platelet microvesicle formation was detectable with FM4-64 and calcein-AM. It is unlikely that these activation-dependent events are similar to what causes lesion, but it was demonstrated that the end point is similar; the insertion of FM4-64 into platelet plasma membrane and an inability to accumulate calcein-AM. Furthermore, phase microscopy of large aggregates indicated that the region of most FM4-64 and least

calcein accumulation coincided with a zone where fibrillar material, presumed to be fibrin was deposited. Examination of aggregates at the electron microscopic level revealed the presence of microvesicles but also platelets that had diluted cytoplasm and gaps in their plasma membrane. It was also noted in some areas that platelets had degenerated to a slurry-like or gel-like material that surrounded activated platelets and membrane-limited structures of unclear identity. This degeneration of platelets has been mentioned before but is poorly described as 'cellular debris'(Jorgensen *et al.*, 1967), suggesting a stochastic rather than an elegant process of degeneration for the apparent purpose of haemostasis. It is submitted that platelet activation beyond a point of no return (i.e. trapped within the core of an aggregate or plug) leads to platelet degeneration and, as such, must be viewed as a form of 'programmed cell necrosis' that has evolved for the deliberate plugging of damaged vessels.

It appears that these dyes are useful for detecting and quantifying loss of platelet viability during storage but also for the detection of microvesiculation / changes to platelets within the medulla of aggregates. Whether the use of FM4-64 for the detection of circulating microvesicles in individuals thought to have high circulating numbers of these entities is feasible remains to be seen. It is worth speculating that the dye when used in conjunction with intravital microscopy would be a useful discriminator of vesiculation events associated with thrombus formation *in vivo*.

4 DEAD PLATELETS FORM AGGREGATES.

4.1 Abstract.

Using calcein-AM and FM4-64 as vital stains it was observed that during *in vitro* storage dead but not live platelets formed loose aggregates. Dead platelets were also CD42b^{-VE}, due to shedding of a 130kDa extracellular fragment into the supernatant. Incubation of platelets with the metalloproteinase inhibitor GM6001 had no effect upon platelet viability but inhibited both the shedding of CD42b and platelet aggregation. The pattern of MMP-2 surface expression changed during platelet incubation and a novel 110kDa gelatinase activity was associated with stored platelets. It was not possible to implicate MMP-2 in either the loss of CD42b or the adhesion event between dead platelets. It is not clear what, if any, physiological relevance this metalloproteinase-dependent adhesion event has but it may be important for clot architecture during haemostatic plug formation.

4.2 Results.

4.2.1 Dead platelets form micro-aggregates during *in vitro* incubation.

Platelets incubated at 37°C as cPRP for several days *in vitro* were observed both by light and electron microscopy to form micro-aggregates. Although many single dead platelets existed, the aggregates that had formed appeared to be populated exclusively by dead platelets according to calcein accumulation / FM4-64 binding. It was rare (<1% of all aggregates examined) to find calcein^{+VE} / FM4-64^{-VE} platelets within the aggregates. By 24 hours of incubation these aggregates commonly comprised of only a small number of platelets, typically between 2 and 30 (fig. 4.1). Further incubation led to the accumulation of greater numbers of dead platelets within the aggregates. During this thesis it was useful to incubate platelets overnight as a 50-fold dilution of PRP; at no time was there any indication that this reduced the amount of death seen by 24 hours relative to incubations done in full PRP. This indicates that platelet death occurred independently of aggregate formation and argues for the formation of aggregates as a result of death rather than vice versa.

4.2.2 Shedding of CD42b is tightly associated with platelet death.

Changes in CD42b are a common feature of storage and activation (Bergmeier et al., 2003; Hoffmeister et al., 2003; Hoffmeister et al., 2003), therefore the surface expression of CD42b was examined during *in vitro* incubation. It was noted that although calcein^{+VE} platelets showed surface expression of CD42b, the epitope recognised by the antibody was completely lost from calcein^{-VE} platelets displaying

morphological signs of death (fig. 4.2.A). To explore the relationship between platelet death and the loss of CD42b platelets were incubated with GM6001, a hydroxamic acid-based inhibitor of various metalloproteinases known to block the loss of CD42b from platelets during storage or due to experimentally induced mitochondrial injury (Bergmeier et al., 2003). It was clear that GM6001 at 100 μ M maintained CD42b expression upon platelets that appeared dead (compare fig.4.2.A.v-viii with fig. 4.2.A.ix-xii).

Bivariate analysis of platelet CD42b expression and calcein accumulation confirmed that only the calcein^{-VE} platelets lost CD42b and that GM6001 was inhibiting this loss without affecting platelet viability (fig 4.2.B). Quantification of the loss of CD42b was performed with platelets over a 5-day period *in vitro* (fig. 4.3.A). The close temporal association of CD42b loss and platelet death did not change during 5 days of incubation, indicating that platelet death did not promote the loss of CD42b from viable bystander platelets.

4.2.3 Surface biotinylation confirms the shedding of a 130kDa protein

Biotinylated proteins shed from the surface of platelets were affinity purified from plasma using streptavidin-coated latex beads and examined by Western blotting. Results indicated that a protein of ~130kDa corresponding to the known size of glyocalicin (Bergmeier *et al.*, 2000), accumulated within plasma during storage. The accumulation of this protein was greatly reduced in supernatants from platelets that had been incubated in the presence of the metalloproteinase inhibitor GM6001 (fig. 4.3.B). These

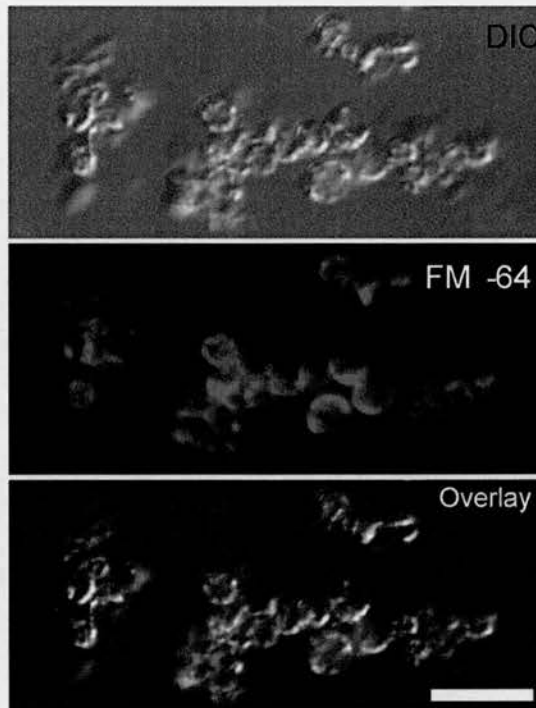


Figure 4.1. Dead platelets associate during *in vitro* storage.

Micrographs show platelets that were aged *in vitro* at 37°C for 72 hours, stained with 5 μ M FM4-64 for 10 minutes and examined by epifluorescence microscopy. Aggregates of various sizes are common by 72 hours but can be seen also at 24 hours. Aggregates appear to be populated exclusively by platelets exhibiting morphological features of death, this is supported by their accumulation of FM4-64. Bar = 10 μ m. Micrographs are representative of images obtained from many different donors.

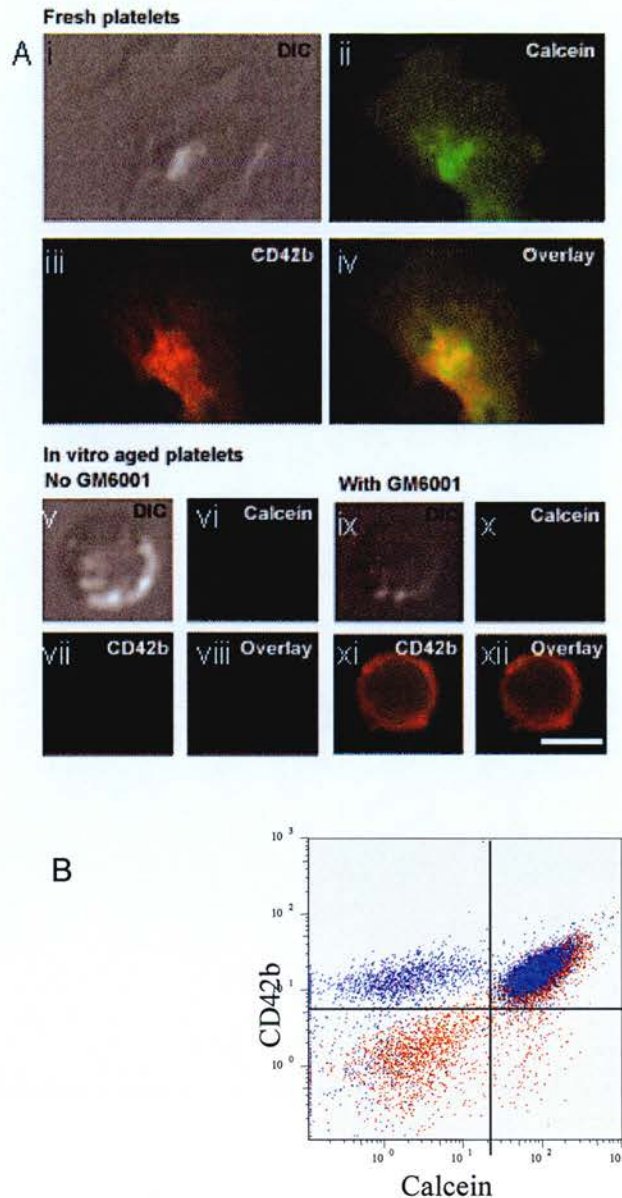


Figure 4.2. Dead platelets lose CD42b.

A freshly isolated platelet incubated stained with calcein-AM and incubated with anti-CD42b antibodies for 20 minutes and then examined by epifluorescence microscopy is shown spread upon glass (A.i-iv). Platelets showing morphological signs of death at 24 hours were unable to accumulate calcein-AM and were noted to be immunonegative for CD42b (A.v-viii). Incubation of platelets with the broad-spectrum metalloproteinase inhibitor GM6001 (100 μ M) inhibited the loss of CD42b from dead platelets (A.ix-xii). Bar=3 μ m. Bivariate analysis of platelets' viability and CD42b expression after *in vitro* incubation for 24 hours (B) confirms that only those platelets unable to accumulate calcein are devoid of CD42b (red plots) and that GM6001 blocks this loss (blue plots) without affecting viability. Micrographs and cytometry plots are representative of data collected from many (>10) donors.

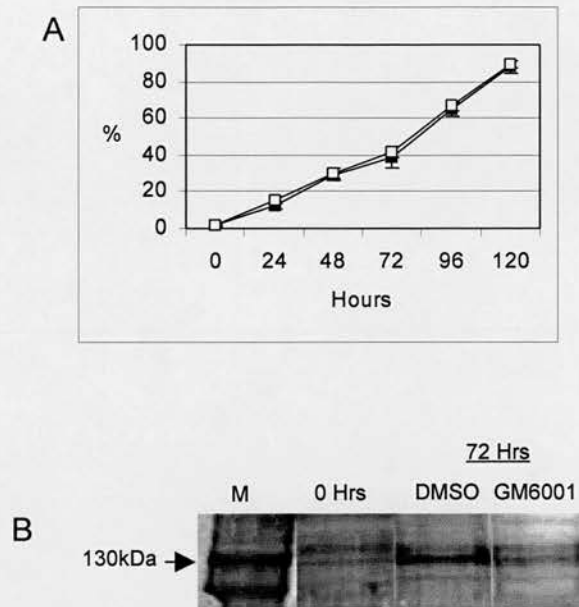


Figure 4.3. Loss of CD42b is closely associated with the timing of platelet death and is inhibited by GM6001.

Platelets were aged as PRP supplemented with magnesium for 5 days and stained with 100nM calcein-AM (open squares) and anti-human CD42b^{RPE} antibodies for 20 minutes (closed squares) and analysed by flow cytometry (A); $n=4$; \pm SEM.

Surface biotinylation allows for the subsequent panning of released biotinylated proteins from platelets with magnetic avidin beads. Platelet proteins captured by the beads were electrophoresed, blotted and probed with streptavidin-HRP. A biotinylated protein of approximately 130kDa corresponding to the expected size of glyocalicin is shed into platelet supernatants over a three-day period but to a lesser extent when GM6001 is present (B).

data indicate that CD42b is shed from dead platelets into the incubation media during storage.

4.2.4 GM6001 has an ED₅₀ of ~20μM.

The inhibitory activity of GM6001 towards the shedding of CD42b has been described at only one concentration, 100μM (Bergmeier et al., 2003), therefore it was of interest to know the effective dose range for the agent. Dose-response data for the ability of GM6001 to inhibit the loss of CD42b from dead platelets was collected from three donors and indicated an ED₅₀ of GM6001 in platelet rich plasma of ~20μM (fig. 4.4).

Examination of platelets stored for 24 hours in the presence or absence of GM6001 showed no difference in the frequency of either calcein^{-VE} or FM4-64^{+VE} platelets between control and GM6001-treated platelets (fig. 4.4.B). Thus GM6001's effect was not due to an indirect ability to affect platelet viability.

4.2.5 GM6001 inhibits the interaction between dead platelets.

The previous data confirm that dead platelets shed CD42b from their surface and that this event appears to be mediated by metalloproteinase activity as evidenced by its sensitivity to GM6001. It was serendipitous then to find that the effect of GM6001 extended also to the aggregate formation between dead platelets. Platelet samples incubated for 72 hours in the presence of GM6001 contained far fewer aggregates than controls (fig. 4.5.A).

As platelet aggregation is observed by an increase in side scatter by cytometry it was reasonable to assume that cytometric analysis of the stored platelets' scatter characteristics would yield an objective measure of aggregate formation and an assay to determine the efficacy of GM6001's ability to block platelet-platelet interactions. Analysis of stored platelets stained with FM4-64 dead confirmed that aggregates (as seen by increased side scatter) were populated by dead and not live platelets. When platelets were stored in the presence of GM6001 the side scatter of dead platelets was qualitatively reduced to that seen with viable platelets (arrows fig. 4.5.B).

4.2.6 The metalloproteinase-dependent adhesion event is independent of plasma factors.

It has been established already that washing platelets from plasma and storing them in calcium-free HBSS for 24 hours at 37°C did not promote death relative to platelets maintained as cPRP (Chapter 3, fig. 3.2). In order to understand the interaction between dead platelets further it was important to establish whether or not soluble plasma factors played a role. It was clear by both microscopy and cytometry that platelets washed from plasma and aged in calcium-free HBSS for 24 hours still formed aggregates, indicating that the adhesion event did not require a soluble plasma factor (fig 4.6.B). Furthermore, aggregate formation was inhibited in washed platelet suspensions by the presence of GM6001, confirming that the metalloproteinase activity responsible for the aggregation was intrinsic to the platelets and not plasma derived. Inhibition of aggregate formation with GM6001 led to maintenance of platelet numbers over a period of 72 hours relative to controls (fig. 4.7).

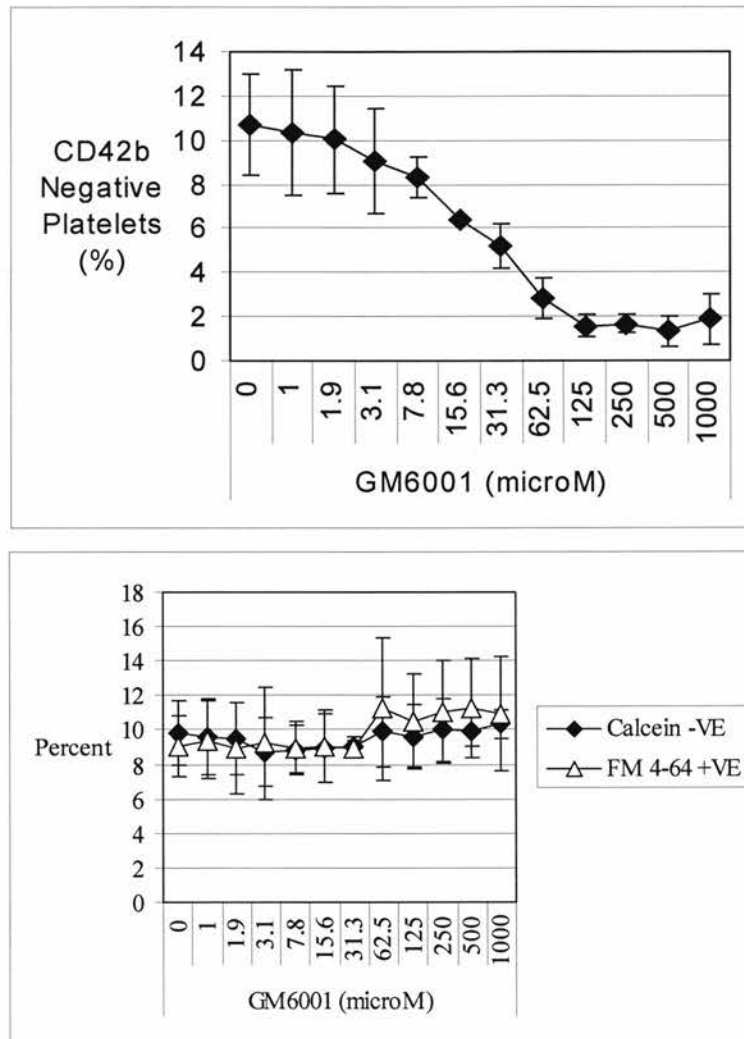


Figure 4.4. GM6001 blocks CD42b loss from platelets with an ED₅₀ of ~20 μ M.

Platelets were incubated for 24 hours at 37°C in either the presence of a broad dose range of GM6001 and examined by cytometry for CD42b expression (A). GM6001 is able to block the loss of CD42b but this is not due to an ability to inhibit platelet death (B). $n=3$; +/- SEM for both graphs.

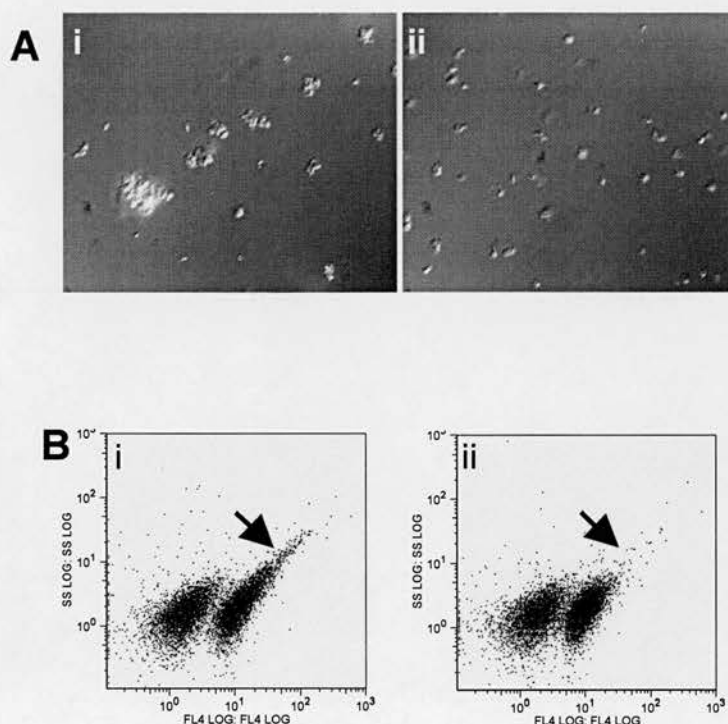


Figure 4.5. GM6001 inhibits aggregate formation between dead platelets.

Micrographs of platelets incubated for 72 hours at 37°C in either the presence (a.i) or absence (a.ii) of 100 μ M GM6001 illustrating the agent's effect on the formation of microaggregates; $n = 3$; \pm SE. Cytometric analysis of side scatter properties confirms that incubation of platelets for 72 hours with the metalloproteinase inhibitor GM6001 abolishes microaggregate formation by dead (FM4-64⁺_{VE}) platelets (arrows in B.i and B. ii). Micrographs and cytometry plots are representative of data collected from >3 different donors.

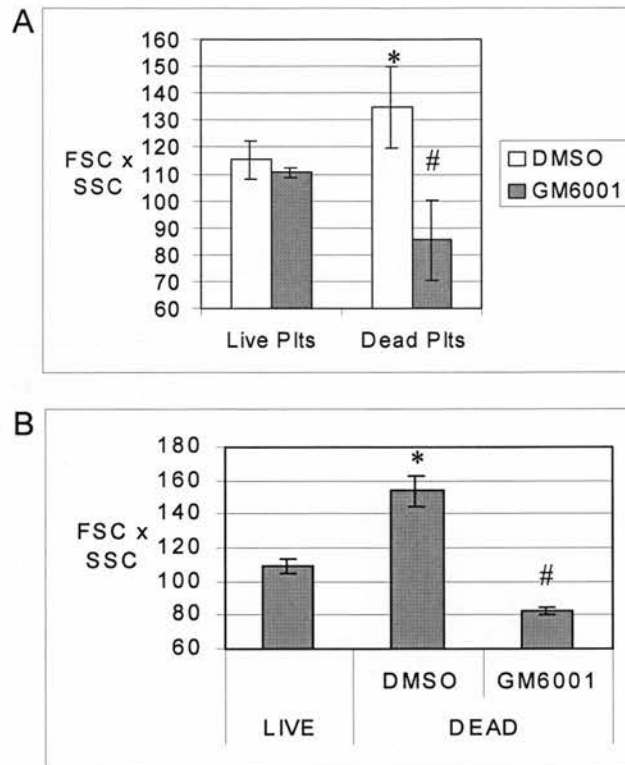


Figure 4.6. GM6001 inhibits aggregate formation between dead platelets.

Platelets were incubated for 24 hours at 37°C in either the presence or absence of 100µM GM6001 and examined by cytometry after vital staining (FM4-64 5µM for 10 minutes). Data is presented as the forward scatter multiplied by side scatter. GM6001 blocks aggregate formation and reveals that dead platelets have reduced scatter properties compared to live platelets (A). $n = 3$; \pm SE. A similar experiment was performed with washed platelets incubated in HBSS to illustrate that no soluble plasma factors are required for aggregate formation (B). $n = 3$; \pm SEM; * $P < 0.05$ from live; # $P < 0.05$ from DMSO.

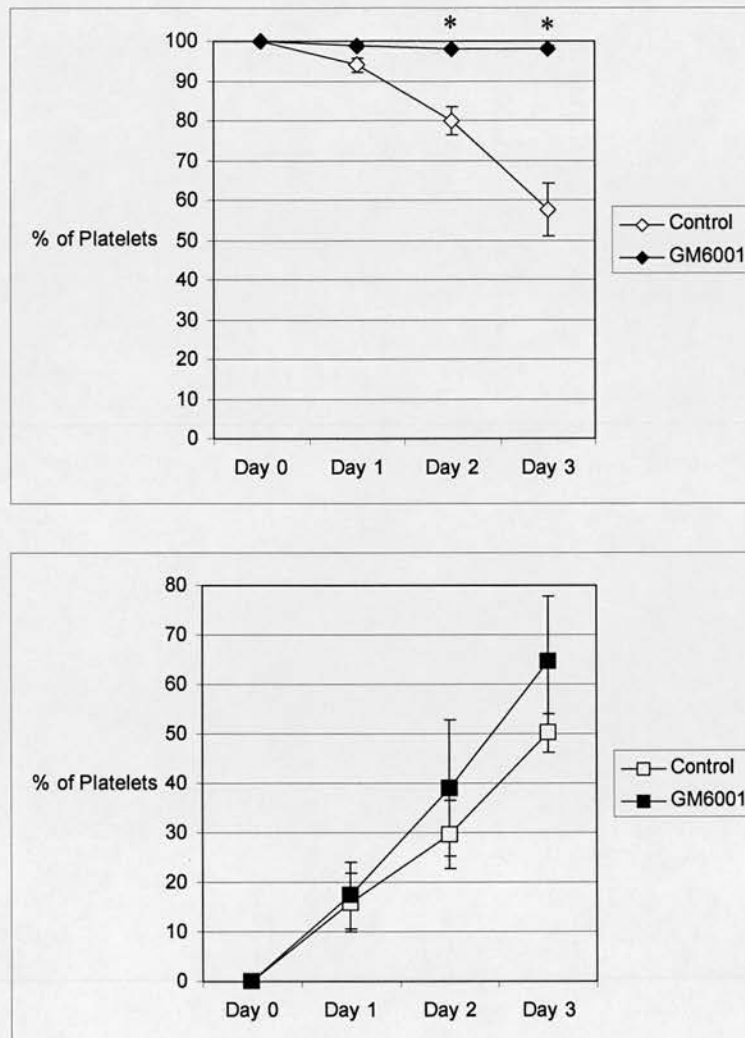


Figure 4.7. Inhibition of platelet aggregate formation maintains platelet numbers and allows more accurate estimate of platelet death rate.

Platelets were incubated as cPRP at 37°C with or without 100µM GM6001 for the length of time as specified in the figures. Platelet numbers were estimated by Coulter Counter and expressed as a percentage of the numbers within freshly isolated PRP. Viability was assessed by cytometry after dual staining platelets with calcein-AM and FM4-64. *n* = 4 different donors; +/-SEM; **P*<0.01 relative to control.

4.2.7 GM6001 fails to affect the aggregation of fresh platelets.

In order to explore the possibility that the adhesion event occurring between dead platelets was a modified version of one that is known to occur between fresh platelets the effect of GM6001 was examined during the aggregation of freshly isolated platelets. GM6001 at concentrations able to inhibit interactions between dead platelets did not affect aggregation of fresh platelets in response to either low or maximal doses of convulxin or TRAP (fig. 4.8), suggesting that its inhibitory target was specific to a death-dependent interaction.

4.2.8 Zymography reveals a novel metalloproteinase activity that is unique to *in vitro* aged platelets.

Proteins from platelets aged *in vitro* for 48 hours were separated by size under non-reducing conditions within acrylamide gel containing 1% gelatin and examined for gelatinase activity. Although enzymatic activity was associated predominantly with proteins of 72kDa corresponding to MMP-2, a minor enzymatic activity was seen also at 92kDa, which corresponds to MMP-9. Of particular interest was the presence of a higher molecular weight gelatinase activity detected at approximately 110kDa in aged but not fresh platelet pellets (fig. 4.9).

4.2.9 Western blotting does not confirm the existence of a 110kDa MMP-2 complex.

To establish whether or not the 110kDa gelatinase activity associated with stored platelets was that of MMP-2, platelet pellets electrophoresed under non-reducing conditions were probed by Western blot with anti-MMP-2 antibodies. Although reactive polypeptides of >200kDa, ~120kDa and ~80kDa could be seen in samples from platelets stored for 48 hours in the presence of GM6001, no other polypeptides were observed in stored or fresh platelet samples (fig. 4.10). The significance of this result is not clear as the antibody (from the same stock) readily detects a 72kDa polypeptide presumed to be MMP-2 under reducing conditions (Simon Riley, *pers. comm.*), thus the antibody may be ineffective for the detection of MMP-2 electrophoresed under non-reducing conditions.

4.2.10 The surface expression of MMP-2 changes during *in vitro* storage.

To further investigate this possibility the surface expression of MMP-2 was examined on stored platelets. After immuno-cytochemical staining stored and fixed platelets it was clear that a broad range of intensity of MMP-2 surface expression was detected. Dual labelling with CD42b enabled the evaluation of platelet viability in the absence of vital stains because CD42b in this work has been shown to be only lost from dead platelets. Four distinct platelet populations could be observed by epifluorescence microscopy. Two viable platelet populations could be seen, one with and one without MMP-2 immuno-reactivity and the same was observed for the non-viable (CD42b^{-VE}) platelets (fig. 4.11). The least complex scenario envisaged by such an expression pattern is that MMP-2 expression is low on freshly isolated platelets but increases several-fold

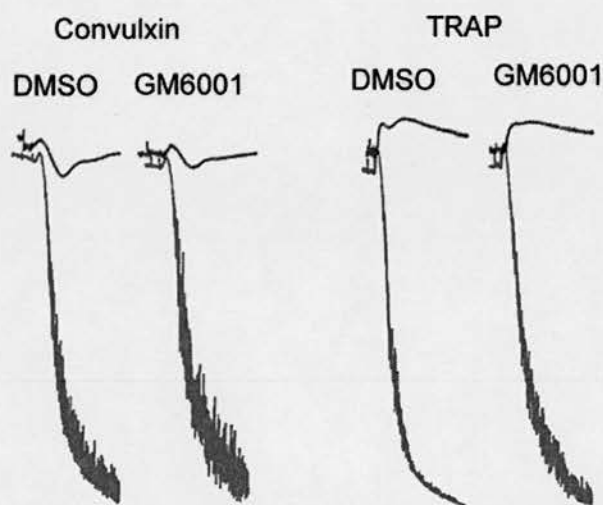


Figure 4.8. GM6001 neither inhibits nor promotes aggregation of platelets in response to CVX or TRAP.

Low and high doses of agonist (green and red plots, respectively) were 10ng/ml or 50ng/ml for Convulxin and 4 μ M or 20 μ M for TRAP. Incubation of fresh platelets for 10 minutes with GM6001 prior to activation (the second group of plots for each agonist) had no effect upon the aggregation of platelets relative to the DMSO control. Plots are representative of experiments carried out with three different donors.

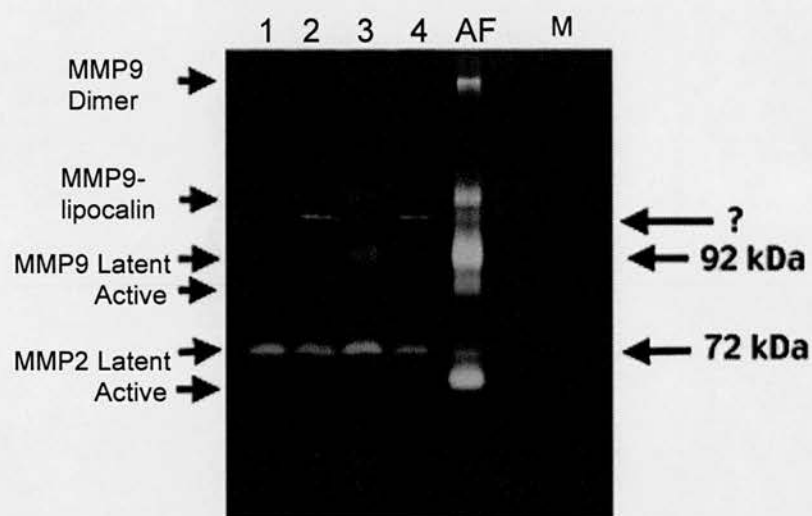


Figure 4.9. Zymography reveals MMP-2 and MMP-9 activity associated with fresh and aged platelets and also a novel activity associated only with platelets stored at 37°C.

Platelets pellets were lysed, electrophoresed through acrylamide gel containing 1% gelatin. The gel was Incubated for 48 hours and stained with coomassie to reveal areas where gelatin had been degraded. Fresh platelets from 2 donors (lanes 1 and 3), platelets aged for 48 hours from the same donors (Lanes 2 and 4). A positive control of amniotic fluid was used to determine MMP activities (AF). M - Markers.

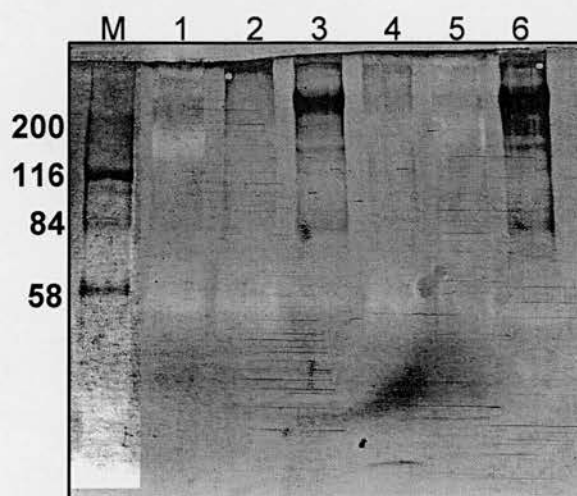


Figure 4.10. A 110kDa MMP-2 polypeptide is not found associated with fresh or aged platelet pellets.

Platelets pellets were lysed, electrophoresed under non-reducing conditions through an 8% polyacrylamide gel, blotted onto a PVDF membrane and probed with anti-MMP-2 antibodies. Lanes 1 and 4 represent samples from 2 different donors' fresh platelets, lanes 2 and 5 are samples from platelets aged *in vitro* for 48-hours and lanes 3 and 6 are from platelets aged for 48 hours in the presence of 100mM GM6001. Immuno-reactive polypeptides corresponding to the expected electrophoretic mobility of MMP-2 activity are not detected, however a large molecular weight polypeptide >200kDa is detected in the samples aged in the presence of GM6001.

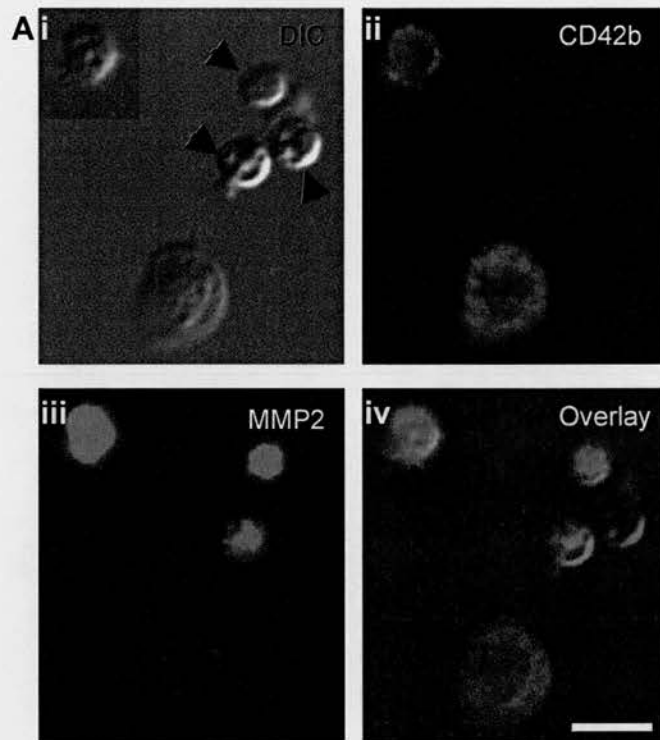


Figure 4.11. MMP-2 appears to be up-regulated on platelets prior to their death and may be lost as a late event.

Platelets were aged for 24 hours at 37°C then fixed with 1 volume of 4% formaldehyde for 20 minutes. Stained with anti-MMP-2 antibodies for 30 minutes at 37°C, washed and incubated with anti-Mouse IgG-Alexa488 for 30 minutes. Platelets were then washed twice and incubated with Mouse anti-CD42bRPE for 20 minutes. An irrelevant primary control IgG (Mouse anti-CD16) gave no staining. Loss of CD42b is indicative of platelet death; three platelets displaying loss of CD42b are shown that also display morphological signs of cell death (arrowheads, i). Two of the dead platelets are MMP-2 immuno-positive, whereas the other is negative. Two live (CD42b+VE) platelets are shown, one with little MMP-2 immuno-positivity the other with considerably more. Bar = 3µm

prior to death and that expression is maintained upon dead platelets until a later event leads to its loss.

4.2.11 The surface expression of CD42b is unaffected by activation with TRAP or incubation of platelets with active MMP-2.

Due to the apparent links between the loss of CD42b, metalloproteinase activity and the interaction between dead platelets it was hypothesised that if a metalloproteinase could be implicated in the modification of CD42b that this would represent a good candidate for the mechanism behind dead platelet-platelet interactions. CD42b expression was examined on fresh platelets and platelets activated with 20 μ M TRAP either in the absence or the presence of serial dilution of active MMP-2. Although MMP-2 bioactivity was confirmed by an ability at 1 μ g/ml to inhibit platelet aggregation in response to either collagen or TRAP (fig. 4.12), it had no effect upon the surface expression of CD42b (fig. 4.13). Nor was there any indication that incubation of fresh platelets with MMP-2 caused spontaneous interactions.

4.2.12 *In vivo* biotinylation of mice enables an estimate of the circulating lifespan of labelled platelets.

Mice were injected with NHS-biotin in order to label circulating blood cells. Examination of whole blood isolated from mice 1 hour after injection of biotin and on several consecutive days revealed that a surface biotinylated population of platelets existed that became reduced in number over the period of analysis (fig. 4.14). The reduction in number of biotinylated platelets was not due to spontaneous loss of surface

biotin because erythrocytes, which are known to circulate for several weeks, displayed no such losses (fig. 4.15).

4.2.13 Injection of mice with GM6001 has no effect upon the circulating lifespan of platelets

The possibility that the adhesion event between dead platelets was a surrogate interaction that would otherwise occur between a dead platelet and a phagocyte was examined *in vivo*. Manipulation of mice by injection either via the intraperitoneal or intravenous route with 15 μ l of 100mM GM6001 was attempted in order to explore the hypothesis that metalloproteinase activity modifies platelets for subsequent recognition and phagocytosis by macrophages. Assuming an average weight of 20-25g per mouse and that this weight correlates with volume, the injection of this much GM6001 was expected to yield a final concentration of approximately 75 μ M; well above the ED₅₀ of 20 μ M estimated for the agent *in vitro*. The curves produced by control and GM6001 treated animals did not differ significantly from each other, regardless of the route of delivery (P=0.876); *n*=3 mice in each group (fig. 4.16).

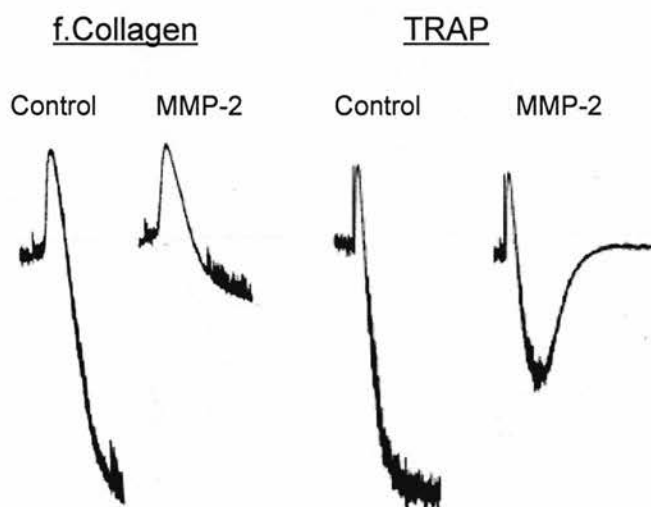


Figure 4.12. MMP-2 inhibits platelet aggregation in response to collagen and also to TRAP.

Platelets were incubated with or without 10ng/ml MMP-2 for 5 minutes prior to the addition of agonist (either fibrillar collagen at 1ug/ml or TRAP at 20μM) and calcium chloride to 2mM. Aggregation proceeded for 5 minutes.

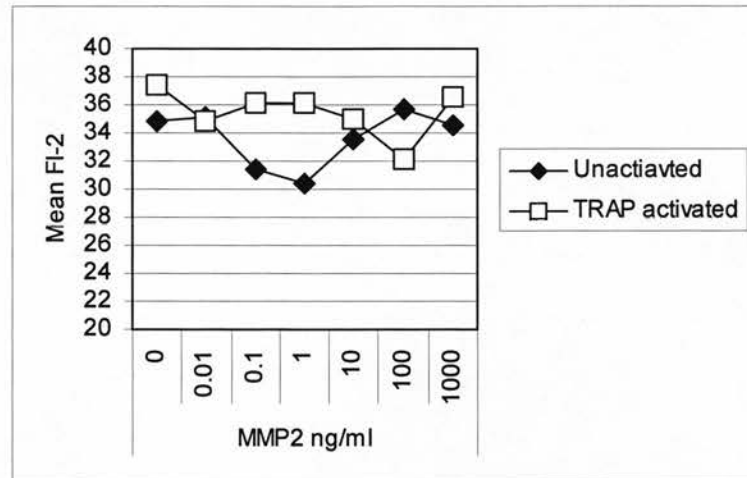


Figure 4.13. MMP-2 has no effect on the surface expression of CD42b on resting or activated platelets.

Freshly isolated platelets were incubated in the presence or absence of 10ng/ml MMP-2, activated with 20 μ M TRAP for 10 minutes and stained with anti-CD42b antibodies and examined by flow cytometry. $n = 1$.

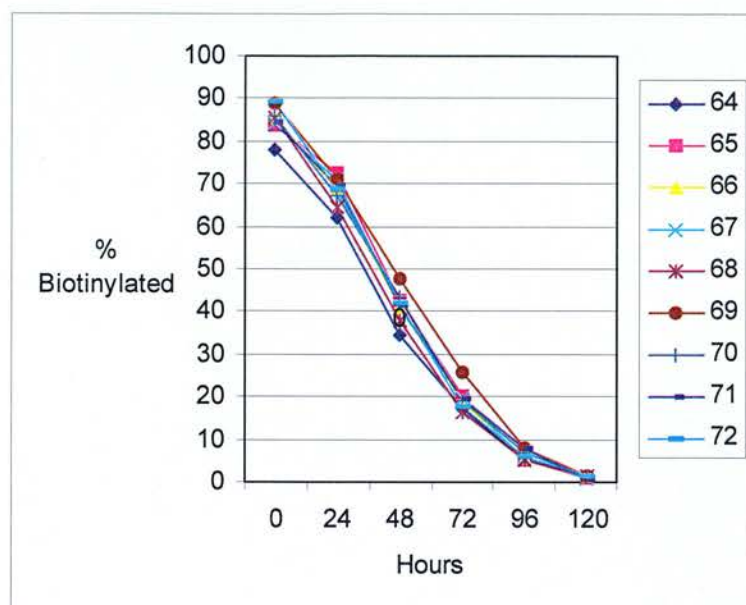


Figure 4.14. Murine platelets can be labelled with biotin by intravenous injection of NHS-biotin.

Nine mice were injected twice (at time -4 hours and 1 hour later) with 6µg of NHS-Biotin in a volume of 200µl of 10% DMSO / PBS. Blood samples were taken at 4 hours after the initial injection (Time 0) from tail nicks into 10 volumes of 3.8% sodium citrate, stained with streptavidin and analysed by flow cytometry for surface biotin against stained blood from non-biotinylated mice. Each line represents the loss of biotinylated platelets from circulation over a 5-day period. The method is highly repeatable with little intra-group variation.

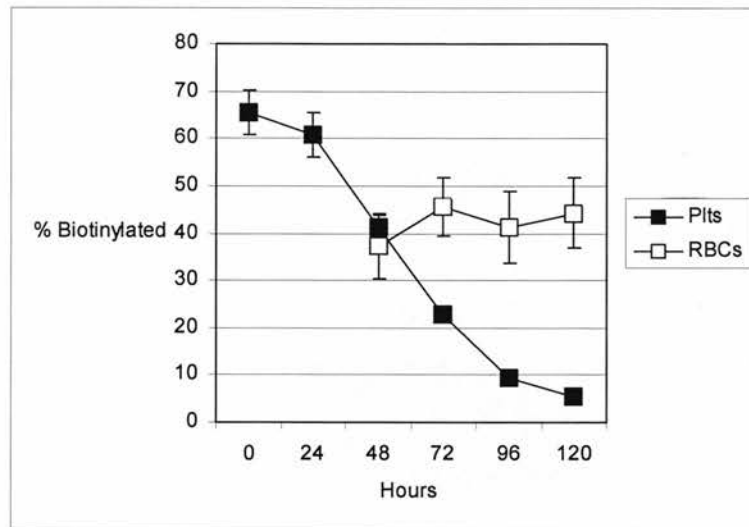


Figure 4.15. Biotin signal is maintained on erythrocytes indicating that it is not spontaneously lost from platelets.

Mice were injected i.v with NHS biotin. Erythrocytes and platelets were labelled using streptavidin and examined by flow cytometry. The loss of biotinylated platelets from circulation does not appear to be due to spontaneous shedding of surface biotin because erythrocytes do not lose the biotin signal despite being within the same environment as the platelets. $n=5$; \pm SE.

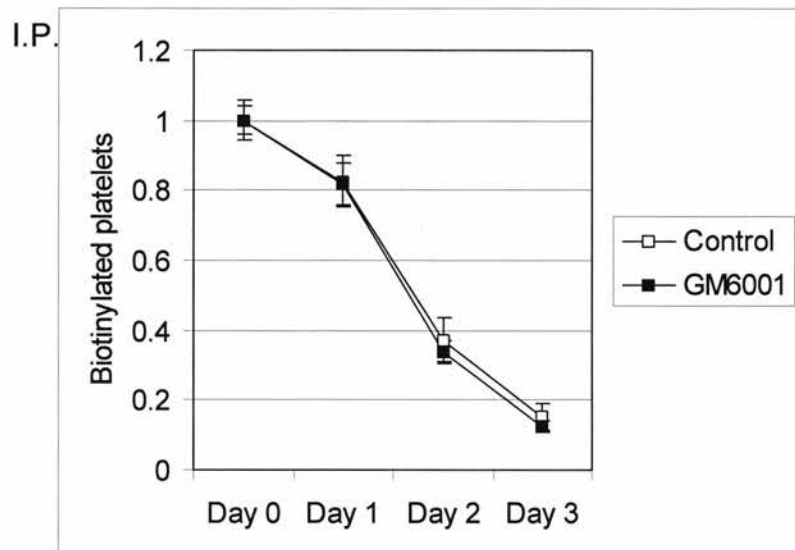
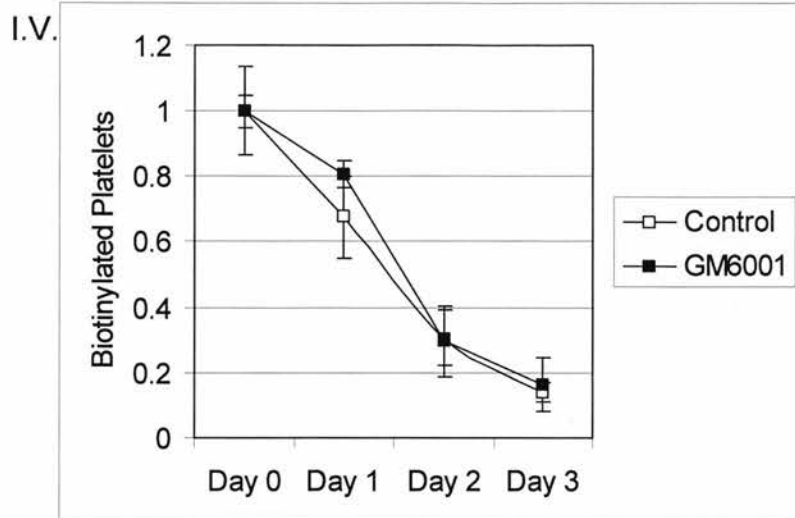


Figure 4.16. Injection of GM6001 into mice does not affect circulating platelet lifespan.

Mice were injected with 15 μ l of 100mM GM6001 dissolved in DMSO (as a bolus of the agent dissolved in PBS) via either an intraperitoneal (i.p) or intravenous (i.v) route on day 0 and day 1. Controls were injected with DMSO:PBS Platelets were labelled on day 0 by *in vivo* biotinylation and blood samples taken on consecutive days to establish the rate of platelet exit from circulation; $n=3$ mice in each group; \pm SE; $P>0.05$ for both treatment routes.

4.3 DISCUSSION.

One of the most obvious consequences of incubating platelets for prolonged periods at 37°C was the formation of micro-aggregates. By both light and electron microscopy it was observed that small numbers of platelets associated with each other within 24 hours of incubation at 37°C and that further incubation led to increased aggregate size.

By electron microscopy it was clear that although single platelets exist with features of cell death the micro-aggregates were comprised of only those platelets that were apparently dead according to their morphology. Use of the vital dyes calcein-AM and FM4-64 in conjunction with antibodies able to identify the α_{IIb} integrin support this conclusion. That platelet death was intimately associated with aggregate formation led to the question as to whether death was dependent upon aggregation or *vice versa*. It was a consistent finding throughout this thesis that the amount of platelet death after 24 hours *in vitro* was similar if platelets had been maintained in cPRP or if platelets had been diluted 50-fold. It is assumed that a 50-fold dilution of platelets is sufficient to reduce the possibility of platelet-platelet interactions and that this result indicates platelet death was not the result of aggregate formation.

A second consequence of platelet death was the shedding of CD42b. Programmed cell death of nucleated cells is associated with loss of surface proteins such as CD31 from apoptotic endothelial cells (Ilan *et al.*, 2001), E-cadherin from apoptotic epithelial cells (Steinhusen *et al.*, 2001) and both CD13 and CD33 from apoptotic THP-1 cells (Brown *et al.*, 1996). Of the small handful of reports regarding this phenomenon the role for

metalloproteinases has been described in the shedding of CD31 and E-Cadherin (Ilan et al., 2001; Steinhusen et al., 2001). Interestingly both cleavage events are mediated by caspase activity upon the cytoplasmic tail of the respective proteins and metalloproteinase activity on the extracellular domain. The significance of these shedding events is not understood but may be something to do with disruption of cell-cell contacts and the exit of the dead cell from its surroundings (Steinhusen *et al.*, 2001) or altered signalling capacity during cell death (Ilan *et al.*, 2001).

Reduced surface expression of vWF receptor proteins (in particular, the CD42b / GPIb α subunit) from platelets has been observed in response to agonists such as thrombin or collagen. The nature of its disappearance has been explained by two models; shedding (Fox, 1994) and translocation to the OCS (Michelson et al., 1991; Michelson et al., 1996), however the authors note that these mechanisms are not necessarily mutually exclusive of each other. It has been observed that CD42b was lost from a subset of platelets during *in vitro* storage and, although the authors do not discuss these platelets explicitly and no mention of aggregate formation was made, it is likely that these platelets represent the same endpoint of the platelets generated in this work (Michelson *et al.*, 1988).

More recently the metalloproteinase-dependent loss of CD42b from both human and murine platelets was demonstrated during *in vitro* storage and in response to experimentally induced mitochondrial damage. Although the authors report almost complete loss of CD42b from both stored and 'damaged' platelets there was no mention of aggregate formation, despite platelets being stored with agitation. Mitochondrial

damage elicited by fCCCP was used as a model of platelet storage lesion, however the work presented in this thesis suggests that platelets without $\Delta\Psi_m$ are not necessarily those that have lost CD42b. However it remains to be seen whether changes to the mitochondria are a better indicator of platelet suitability for subsequent transfusion than loss of viability as assessed by either the calcein or FM4-64 assays. It is not clear why in the current work the loss of CD42b did not correlate with loss of $\Delta\Psi_m$ according to JC-1. The deliberate collapse of $\Delta\Psi_m$ in human and murine platelets with high concentrations of fCCCP (100 μ M), may have been somewhat aggressive and unnecessary method of inducing storage lesion (considering that PSL occurs spontaneously). The data presented by the authors and by the same group in a more recent paper indicates that platelets were activated by the fCCCP treatment (Bergmeier et al., 2004), thus the loss of CD42b from the majority of platelets may have reflected indirect effects of rapidly and massively damaging mitochondria, which are a large source of calcium (Rizzuto *et al.*, 2000). Nevertheless, the work presented in this thesis agrees with previous findings indicating that stored platelets lose CD42b due to metalloproteinase activity.

Despite a number of groups documenting the accumulation of spontaneous aggregates during platelet storage and reduction of platelet numbers during storage (Murphy and Gardner, 1971; Holme et al., 1987; Martin-Valmaseda et al., 1999) an interaction between dead platelets has not been described before. This is possibly due to the entirely reasonable assumption that the aggregates had formed due to platelet activation. The mechanism of adhesion between dead platelets appears not to be common to those induced by activation of fresh platelets because a) GM6001 had no effect upon the

aggregation of fresh platelets. This may indicate that the adhesion event is dependent upon platelet death and thus represents a novel platelet-platelet interaction of unknown physiological relevance.

Due to the apparent metalloproteinase-dependent nature of both CD42b shedding and the adhesion event between dead platelets it was important to examine metalloproteinase activity associated with stored platelets. Gelatin zymography is known only to identify MMP-2 and MMP-9 activities (Simon Riley, *pers. comm.*). Typically, latent MMP-2 and 9 activities are observed at 72 and 92kDa, respectively with proform activity being seen at approximately 10kDa less due to loss of the inhibitory N-terminal pre domain. Zymograms consistently show other activities at >200kDa and 125kDa which are thought to be due to MMP-9 dimerisation and MMP-9 complexed with a member of the lipocalin family. In the present work gelatinase activity associated with platelet pellets complied with this expected pattern of activities and, for resting platelets, was the same as that published by others (Fernandez-Patron *et al.*, 1999). However, a gelatinase activity of approximately 110kDa was associated with the pellets of stored platelets and, to the author's knowledge, has not been described for platelets. An extensive literature search and conversation with researchers in the field of metalloproteinase biology indicates that only a single report exists regarding a 110kDa gelatinolytic activity (Kim *et al.*, 2001). This activity was seen in bovine follicular fluid and was described as being a novel form of MMP-2 due on the basis of western blotting evidence. Although the 110kDa gelatinase activity was not identified in the present work as MMP-2 further attempts should be made to confirm its identity because it is the only activity unique to stored platelets and may represent an MMP or MMP-containing

protein complex responsible for CD42b shedding and the interaction between dead platelets.

Immunocytochemical analysis of platelets with anti-MMP-2 antibodies revealed that the surface antigenicity of this metalloproteinase changed dramatically during *in vitro* storage. Dual staining with anti-CD42b antibodies confirmed previous results that loss of CD42b coincided with dramatic changes to platelet morphology associated with death and that the surface expression of MMP-2 appeared to increase upon viable platelets during storage. Dead (CD42b negative) platelets showed low or high-level surface expression of MMP-2; the simplest scenario to explain this temporal pattern of expression is that MMP-2 surface expression is greatly up regulated prior to platelet death but at some point *after* death its antigenicity is lost from the platelet surface. Due to the complexity of MMP regulation and activity it is very difficult to speculate as to the relevance of this change in MMP-2 expression to the changes associated with dead platelets thus further examination of the effects of MMP-2 on platelets was sought.

In an attempt to implicate or exclude MMP-2 in the loss of CD42b and the interaction between dead platelets freshly isolated platelets were incubated with active MMP-2. Addition of exogenously activated MMP-2 to PRP failed to effect spontaneous platelet-platelet interactions nor changed the surface expression of CD42b; the latter of these results contradicts results gained by others indicating that MMP-2 can positively affect CD42b expression on platelets (Radomski *et al.*, 2001). The reasons for this discrepancy are unclear, especially as MMP-2 bioactivity was confirmed by its ability to inhibit platelet aggregation in response to collagen or TRAP.

The incubation of single purified metalloproteinases with platelets as a means of implicating a particular species is flawed inasmuch that it is entirely plausible that the MMP activity responsible for the observations made in this thesis relies upon, and is subsequent to, other signals / events within dying platelets. For example, the temporal pattern of MMP-2 surface expression does not correlate with CD42b shedding – suggesting that either the metalloproteinase is not involved with loss of CD42b / the adhesion event or that its activity is not sufficient to bring about these changes. This is certainly true for the shedding of CD31 from endothelial undergoing apoptosis; the cytoplasmic tail of CD31 appears to require proteolysis by a caspase before metalloproteinase activity could liberate a soluble form of the protein from the extracellular domain (Ilan *et al.*, 2001). Thus, the incubation of active MMPs with fresh platelets may be an inadequate way of establishing which MMP effects the interaction between, and the shedding of CD42b from, dead platelets.

An unanswered but key question raised by this thesis is what, if any, physiological role is there for either platelet death or the metalloproteinase activity mediating an interaction between dead platelets? MMP-2, like many other MMPs, has a vitronectin-like domain recognised by the vitronectin receptor ($\alpha_v\beta_3$ integrin complex) (Silletti *et al.*, 2001) and it is known that the recognition of apoptotic cells by macrophages can be mediated by the macrophage vitronectin receptor (Savill *et al.*, 1990) thus it is worth speculating that the change in MMP-2 expression upon the platelets' surface may aid recognition of effete platelets by macrophages via their vitronectin receptors prior to their death (as assessed by calcein or FM4-64). To address this possibility mice were

injected with GM6001 and the agent's effect upon the circulating lifespan of platelets was assessed by *in vivo* biotinylation. No effect on the platelets' lifespan was recorded, however it is difficult to interpret this data without knowing if the GM6001, once injected, had retained biological activity in circulation. Injection of mice with the agent and subsequent analysis of their blood plasma for its ability to inhibit loss of CD42b from human platelets during storage would resolve this question. Furthermore, it may be expected that analysis of the circulating lifespan of platelets as assessed by biotinylation would not report on changes to the mechanism of removal of effete platelets from circulation because no information is obtained about retention time of such platelets within the spleen prior to phagocytosis by splenic phagocytes (if indeed this is the means by which platelets are removed from circulation). Thus if bioactivity was confirmed without an effect upon lifespan this could be a false positive result. In the event of a positive result (an extension of platelet lifespan due to failed clearance of effete platelets) an extensive examination of splenic architecture would have to be performed to establish if results were due to indirect effects of the agent on numbers, viability and function of phagocytes within the spleen.

At the time of writing this thesis a paper was published that implicated strongly the metalloproteinase activity of TNF-alpha converting enzyme (TACE) in the shedding of CD42b from platelets (Bergmeier et al., 2004). The authors demonstrated that plasma glyocalicin levels in TACE-deficient mouse are reduced by 90% compared to controls and appear to have solved the decades-old mystery of which enzyme controls glyocalicin levels within blood. Interestingly there does not appear to be any differences between TACE-deficient platelets and controls in terms of size, number,

aggregation, degranulation or tail bleeding times. In agreement with the group's earlier publications the post-transfusion recovery of TACE-deficient platelets was improved relative to controls. It still remains unclear what the physiological significance the shedding of glyocalicin has. Storage of platelets from the TACE-deficient mouse would help resolve whether the interaction between dead platelets is due to TACE activity.

Interestingly dead platelets like those generated *in vitro* could not be found within murine spleens, despite extensive searching (see appendix, fig. 8.4). This result reflects a general frustration that platelet-phagocyte interactions were rarely explicitly presented by any groups within the literature. Obviously the absence of a result in no way indicates a negative, however it highlights a difficulty in the establishment of a clearance mechanism *in vivo*. If phagocytosis is the means by which platelets are removed from circulation the treatment of spleens with lysosomal inhibitors to cause an accumulation of material within phagolysosomes and outwith the phagocytes could be employed to study the phenomenon; a strategy analogous to the methodology used in the study of autophagosome formation (Yamamoto *et al.*, 1998).

In summary, platelet death *in vitro* involves a metalloproteinase dependent process of CD42b shedding and an interaction between dead platelets. An individual metalloproteinase activity could not be implicated but changes to MMP-2 surface expression occurred during *in vitro* storage. Furthermore a novel gelatinase activity was associated with stored but not fresh platelets. It is not clear what the interaction or the shedding events represent nor what physiological relevance they have. It is difficult to

find platelets exhibiting signs of death in spleens but they are readily found within aggregates generated *in vitro* (this study) and *in vivo* (Jorgensen et al., 1967; Davies et al., 1975). In view of the data regarding the differential accumulation of calcein and FM4-64 in collagen-induced aggregates, the possibility arises that changes to platelets occurring within aggregates and haemostatic plugs may be synonymous with death and that metalloproteinase activity stabilises the architecture of these developing structures.

5 AUTOPHAGY DURING PLATELET DEATH

5.1 Abstract.

Platelets die *in vitro* in a caspase independent manner with features of necrosis. Therefore the role of an alternative cell death path for human platelets was examined. By electron microscopy it was observed that large vacuoles formed within platelets during 24 hours of storage at 37°C. Vacuoles contained amorphous material of uncertain origin and were sometimes lined with electron dense granular material presumed to be glycogen. By epifluorescence microscopy platelets could be seen to have accumulated both lysotracker red and the autophagic vacuole marker monodansylcadaverine to discrete small structures within freshly isolated platelets but to large vacuoles within stored platelets. The large vacuoles that formed within stored platelets showed positive immune staining for Lamp-2, confirming their lysosomal origin. Storing platelets in the presence of amino acids or the autophagy inhibitor 3-methyladenine incompletely inhibited vacuole formation. 3-MA was toxic to platelets. Amino acids did not promote survival but did promote the retention of platelet function. Concomitant with the increase in large vacuoles was the reduction of α_{IIb} surface expression. The loss of α_{IIb} and the formation of vacuoles were inhibited by the supplementation of 2mM magnesium sulphate. 3-MA was not toxic to platelets in the presence of $MgSO_4$. Platelet death *in vitro* does not appear to be due to a classical autophagic process although it may represent an adaptation to low-cation conditions for the preservation of function.

5.2 Results.

5.2.1 Vacuoles appear in stored platelets.

Electron micrographs of platelets indicated the presence of large vacuoles within stored but not fresh platelets that had retained discoid shape and were not showing signs of cell death such as cytoplasmic condensation and loss of membrane integrity (see Chapter 3). It has been established in the previous chapter that *in vitro* platelet storage does not lead to classical signs of activation (i.e. integrin activation and CD62P expression due to degranulation) thus the vacuoles appear independently of the platelets' activation status. By transmission electron microscopy, vacuolisation appeared to be restricted to the formation of one to two large vacuoles per platelet. Vacuoles showed heterogeneous morphology, some were lined with glycogen (fig 5.1, arrow in A and B), some contained amorphous material of unclear origin (fig 5.1, arrows in C and D). To the periphery of many vacuoles were small double membrane limited vesicles dissimilar from alpha and dense granules, which are relatively larger and more electron dense (fig 5.1, arrow D). However, at no point were multi-lamellar macro-autophagic vacuoles observed. Apposed membrane regions existed in some platelets but were not seen connected to vacuoles (fig 5.1, arrowhead in A, B and E).

5.2.2 Vacuoles accumulate Lysotracker red and are immuno-positive for Lamp-2.

It was reasonable to assume that, because the vacuoles contained what appeared to be degraded material, they were lysosomal in origin. It was pertinent also to examine by a less labour intensive method than electron microscopy the formation of these vacuoles

during *in vitro* storage of platelets. To confirm the identity of these vacuoles as lysosomes the acidotropic dye LysoTracker red (LTR), was incubated with platelets. The dye accumulated rapidly (within 10 minutes at 37°C) in both fresh and aged platelets to discrete structures (fig 5.2.A and B), and there was accumulation also of the dye to the periphery of large structures in aged platelets (fig 5.2, arrow in B). Incubation of fixed and permeabilised platelets with antibodies to Lamp-2, (an integral lysosomal membrane protein important for lysosomal development and the docking of autophagic vesicles (Eskelinen *et al.*, 2003)) confirmed that the vacuoles were lysosomal in origin (fig 5.2.C).

5.2.3 Lysosomes and large lysosomal vacuoles accumulate monodansylcadaverine (MDC).

MDC is an autofluorescent lipophilic molecule that has been shown to specifically accumulate within autophagic vacuoles at various stages of their development. It has been demonstrated in various mammalian cell lines that MDC accumulates to small spherical structures that, when fractionated through sucrose gradients and examined by TEM, have morphology consistent with them being autophagic vacuoles (Biederbick *et al.*, 1995). The dye has been used also to identify autophagic vacuoles that formed in Chinese hamster ovary (CHO) cells in response to amino acid starvation (Munafo and Colombo, 2001). When incubated with fresh or aged platelets MDC co-localised to the same structures as LTR (fig 5.2). Although there was reason to believe that MDC accumulated with varying efficiency to different LTR^{+VE} structures this was not quantified. These data indicated that MDC was capable of labeling lysosomes in

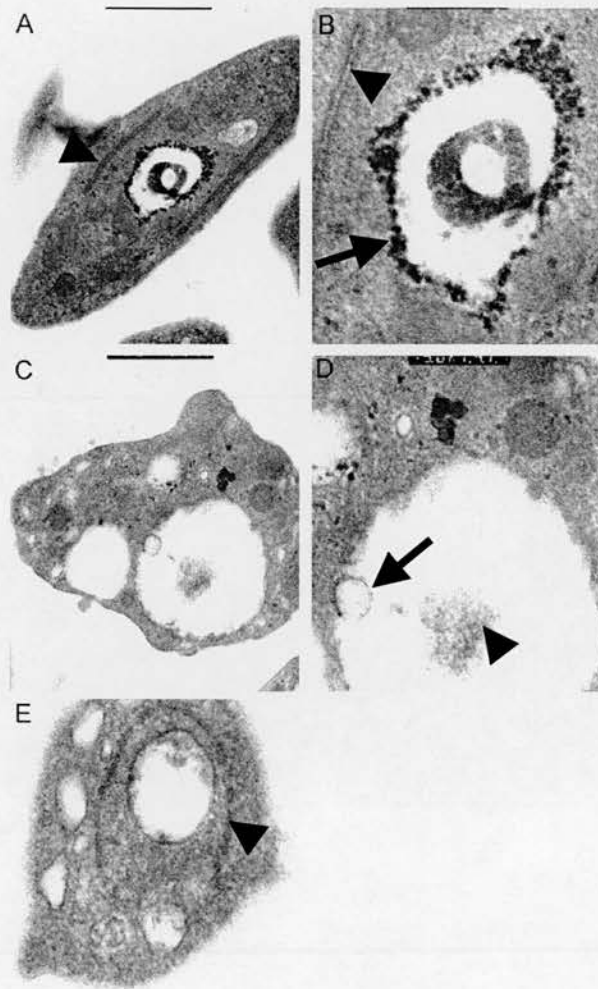


Figure 5.1. Vacuoles appear in platelets aged *in vitro* at 37°C.

Electron micrographs showing platelets aged for 24 hours containing large vacuoles lined with electron dense material consistent with the appearance of glycogen (arrow, B). Various vesicle structures (arrow, D) are associated with vacuoles with internal amorphous contents (arrowhead, D). Open canalicular system zippering can be seen (arrowheads A, B and E).

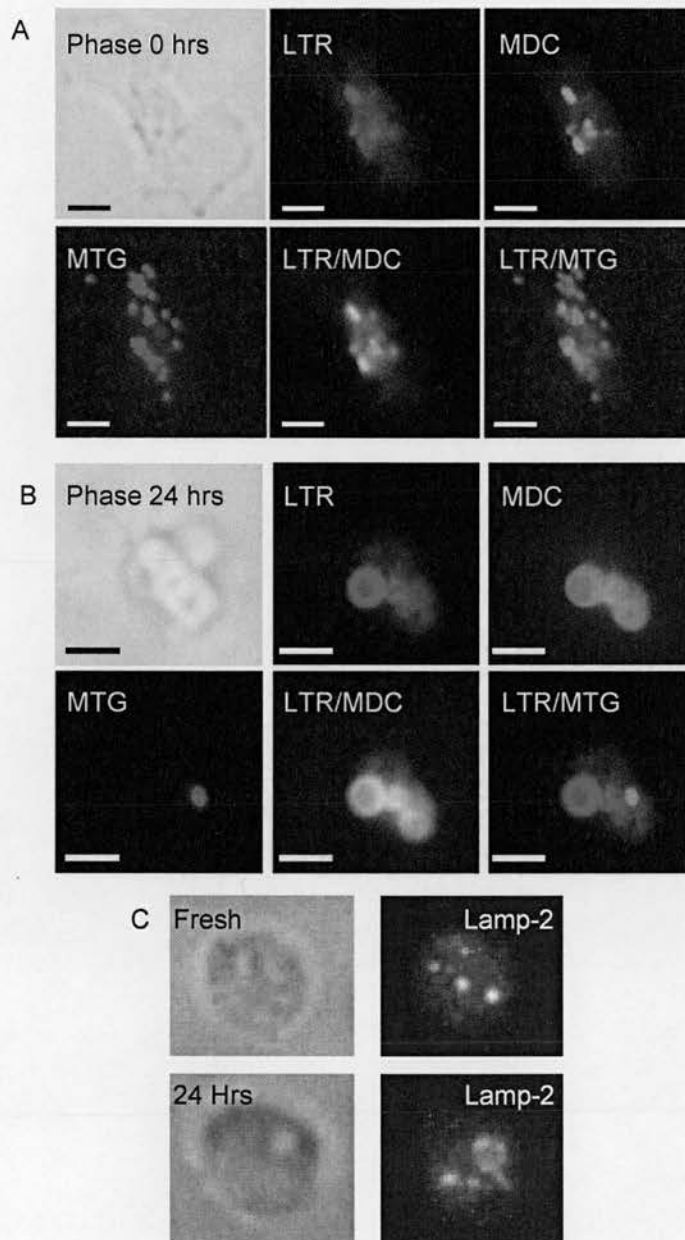


Figure 5.2. Platelets accumulate Lysotracker red, mito-tracker green monodansylcadaverine and are immunopositive for Lamp-2.

Platelets both fresh and incubated for 24 hours accumulated LTR, MTG and MDC to discrete structures within the cytoplasm. MDC and LTR appear to colocalise without exception. MTG accumulates within discrete structures distinct from those labelled by LTR or MDC. Large vacuoles that form during incubation accumulate LTR and MDC but do not accumulate MTG. Immuno-positive staining for the lysosomal protein Lamp-2 can be seen in small structures within fresh platelets and within large vacuoles of in vitro aged platelets (C).

platelets and that it is not a specific marker of autophagic vacuoles in these cells, however this does not exclude the possibility that the large LTR^{+VE} / MDC^{+VE} vacuoles were autophagosomes.

5.2.4 Lysosomal vacuoles are rare in fresh platelets but relatively common in aged platelets.

It was clear by both electron and light microscopy that vacuolisation of platelets was occurring in viable platelets prior to death; therefore quantification of the frequency of platelets containing these vacuoles was performed only on the platelets able to spread on glass and did not include dead platelets. Dead platelets could not be assessed for vacuoles due to the state of their cytoplasm and loss of organelle structure.

No evidence of a loss of vacuoles during spreading was observed. Of the freshly isolated platelets able to spread on glass only 1.4 +/- 0.2% contained large vacuoles whereas 38.4 +/- 2.2% of the platelets that had been aged overnight had vacuoles (fig 5.3.A).

5.2.5 The autophagy inhibitor 3-Methyadenine (3-MA) partially blocks the formation or enlargement of vacuoles.

3-MA was identified as an inhibitor of autophagy in 1982 during a screen for compounds able to inhibit lysosomal proteolysis but not protein synthesis (Seglen and Gordon, 1982). It is used widely in the autophagy field and is one of the few tools able

to interfere with the autophagic process. It would be expected therefore that if vacuolisation was autophagic in nature 3-MA could inhibit the process.

Incubation of platelets in the presence of 10mM 3-MA led to a 53% reduction in the frequency of platelets containing large vacuoles (overnight control, 38.4 +/- 2.2%; overnight in the presence of 10mM 3-MA, 17.0 +/-1.4%; $n=4$, $P<0.01$) (Fig 5.3.A), consistent with the notion that vacuolisation represented an autophagic process. However, concomitant with the partial inhibition of vacuole formation during storage was an increased frequency of dead platelets indicating that 3-MA was also toxic, suggesting that vacuolisation might be less to do with an active death program in platelets and more to do with a survival process, that if inhibited, contributed to cell death (fig 5.3.B).

5.2.6 Amino acid supplementation reduces vacuole formation.

To study the possibility that platelets were adapting to a nutrient-limiting environment by up-regulating autophagy platelets were supplemented with 1x amino acids and aged for 24 hours at 37°C. Amino acid supplementation of platelets significantly reduced vacuole formation by 57% from control levels (control platelets, 38.4 +/-2.2%; amino acid supplemented platelets 16.3 +/-2.4%; $n=4$, $P<0.01$) (fig 5.3.A). Although amino acids were able to reduce vacuole formation there was no concomitant pro-survival effect elicited by the supplementation, contrasting the data obtained with 3-MA that suggested vacuolisation was a survival process (fig 5.3.B). This indicates that amino acids, although able to block vacuolisation, do not affect platelet viability and that

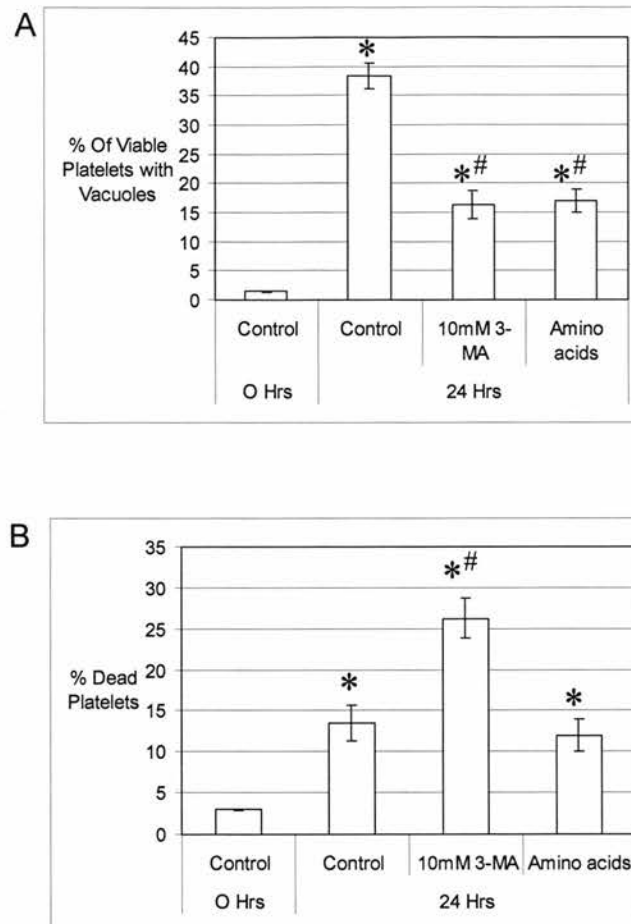


Figure 5.3. Vacuoles are rare in freshly isolated platelets but commonly seen in platelets aged overnight.

Platelets were aged overnight in the presence or absence 10mM 3-MA, amino acids or without supplementation. Platelets were assessed for the presence of vacuoles (A) and results were expressed as the ratio of platelets with vacuoles versus those without. Viability (B) was assessed with 100nM calcein-AM and it was found that by 3-MA negatively affected viability and correlated with incomplete inhibition of vacuole formation. Amino acids although inhibiting vacuolisation had no effect on viability. $n > 4$; +/-SEM; * $P < 0.01$ from Time 0; *# $P < 0.01$ from control at 24 hours.

vacuolisation is thus uncoupled from survival. This apparent uncoupling argues also that the effects of 3-MA on vacuolisation are due to toxic effects upon platelets rather than any direct ability of the drug to interfere with autophagy.

5.2.7 Fresh and *in vitro*-aged platelets accumulate similar amounts of MDC.

To examine the possibility that the large vacuoles were forming *de novo* (as might be expected if macro-autophagy was the driving force for their production) the amount of MDC-associated fluorescence in platelets that had been incubated with the dye for 20 minutes at 37°C was quantified by flow cytometry (fig. 5.4). Fresh platelets and *in vitro*-aged platelets exhibited similar levels of MDC fluorescence (40.9 \pm 3.8mfu and 36.5 \pm 3.4mfu, respectively; $n=4$; $P=0.11$) and incubation of platelets with amino acids gave similar results (45.4 \pm 6.1 and 44.8 \pm 5.6; $n=4$; $P=0.90$). The presence of 3-MA reduced MDC accumulation within platelets by ~45% from 40.1 \pm 4.0 when fresh to 21.9 \pm 2.0mfu after 24 hours ($n=4$; $P<0.01$). This effect was not due to 3-MA interfering with MDC fluorescence because 3-MA was present within the fresh platelet samples. It was unlikely that this reduction in the ability of platelets to accumulate MDC was the result of increased platelet mortality because MDC fluorescence was reduced within the whole population of platelets. This data indicated that in control and amino-acid supplemented platelet samples neither a loss nor gain in MDC accumulation occurred as a result of platelets being aged *in vitro* for 24 hours, whereas ageing platelets in the presence of 3-MA had a profound negative effect upon the platelets' ability to accumulate the dye.

5.2.8 Amino acids maintain platelet function after storage and 3-MA

completely inhibits platelet activation in response to convulxin.

Due to amino acid supplementation having no effect upon platelet survival it was postulated that the apparent autophagy was capable of 'eating into' non-vital components of the platelets, such as storage granules. To this end it was worth exploring the platelet's functional response to the strong agonist, convulxin (fig 5.5). Freshly isolated platelets supplemented with amino acids had improved degranulation in response to CVX when compared to controls (61.1 \pm 8.2% control; 76.2 \pm 4.3% amino acid supplemented; $n=4$; $P<0.05$). Interestingly, 3-MA within the same experiment completely inhibited degranulation (1.9 \pm 0.4%) (and also shape change as assessed by cytometry). The same experiment performed 24 hours later after *in vitro* storage revealed that amino acids supplemented platelets retained a greater ability to degranulate (relative to the amount they degranulate when fresh). Control platelets' response to CVX was reduced by 61% (to 26.8 \pm 8.44 % of platelets expressing CD62P), whereas platelets maintained overnight with amino acid supplementation showed only a 30% reduction in their ability to degranulate (52.0 \pm 5.8% expressing CD62P) ($n=6$; $P<0.01$). In all non-activated controls at 24 hours no CD62P expression was observed confirming that platelets do not spontaneously degranulate alpha granules during *in vitro* storage. This result is consistent with the idea that vacuolisation may involve a degradation of granule contents in the lysosome and that amino acids are capable of blocking this process without affecting platelet viability.

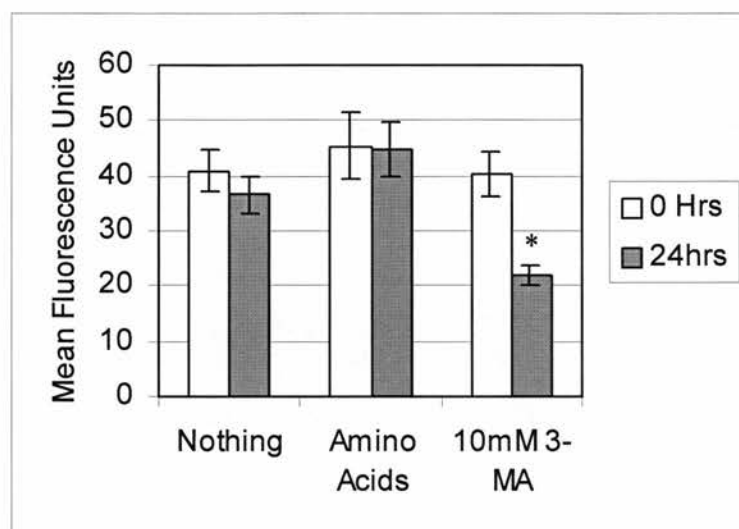


Figure 5.4. Changes to platelet-associated monodansylcadaverine fluorescence (MDC).

Fresh platelets incubated supplemented with 1x amino acids or 10mM 3MA were incubated with 25 μ M MDC for 10minutes and analysed for fluorescence by cytometry. MDC staining was performed again after platelets had been incubated at 37°C for 24 hours. Fresh and *in vitro* aged platelets incubated in the presence of amino acids accumulated MDC similarly, however, platelets aged in the presence of 10mM 3-MA, failed to accumulate MDC to similar levels relative to freshly isolated. $n=4$; \pm -SEM; * $P<0.01$.

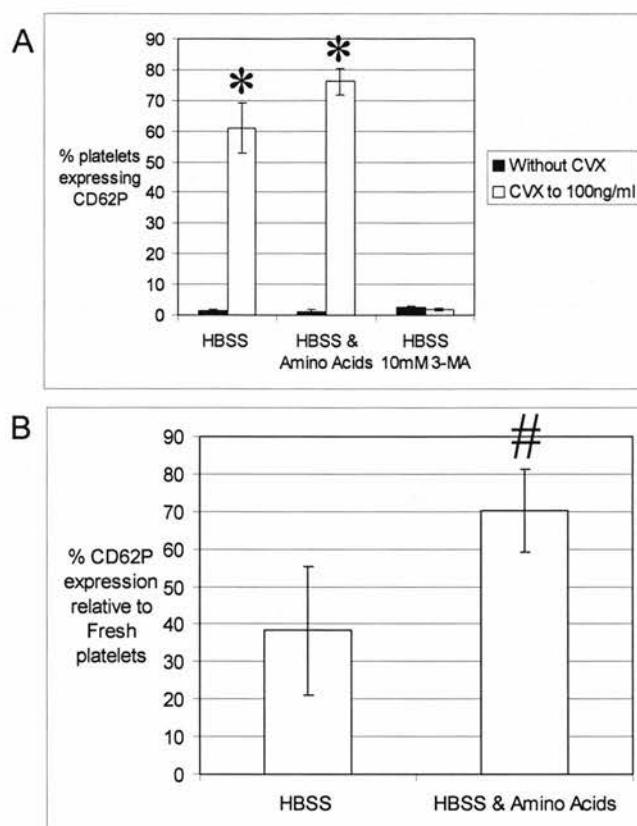


Figure 5.5. The effects of amino acids and 3-MA on platelet function before and after *in vitro* storage.

Platelets were activated with 50ng/ml convulxin (CVX) in the presence of 2mM CaCl_2 when fresh (A) and 24 hours later (B). Activation was determined by the level of degranulation as assessed by the percentage of platelets expressing CD62-P and are depicted as percentage platelets expressing CD62P (A) or the percentage of platelets expressing CD62P relative to the results obtained when fresh. $n = 6$; \pm SEM; * $P < 0.01$ compared to non-activated controls; # $P < 0.05$ vs HBSS.

5.2.9 Mitochondria are readily identified in platelets using Mitotracker Green.

The observation of lysosomal autophagic-like vacuoles led to the question of what intra-platelet components were being trafficked to the vacuole for destruction. Although no discernible intraplatelet organelles could be identified at the TEM level it was hypothesised that, because of the lack of effect that Antimycin had on platelet viability and the uncoupling of a loss of $\Delta\Psi_m$ from viability, damaged mitochondria were being destroyed in the lysosomes. This phenomenon is known to occur in neurons starved of growth factors (Xue *et al.*, 1999) and is thought to result in the specific destruction of damaged mitochondria by autophagy (Elmore *et al.*, 2001).

Mitotracker green is able to accumulate with very little cytosolic background fluorescence in mitochondria and the dye accumulated to discrete structures in platelets that were distinct from lysosomes and dense granules as assessed with LTR and quinacrine (not shown), respectively (fig. 5.6). To the authors knowledge this is the first usage of the dye with platelets and revealed that mitochondria exist as apparent single entities within platelets in contrast to their 'string of beads' appearance in other cells. This singleton morphology may represent their low level of activity within resting platelets.

The number of mitochondria in fresh platelets that were allowed to spread was 9.27 ± 0.51 per platelet; this number decreased to 7.1 ± 0.3 mitochondria per platelet after 24 hours incubation ($n=5$; $P<0.01$) (fig 5.5, A).

5.2.10 Viable platelets have a decreased ability to accumulate NAO.

Because MTG is thought to be sensitive to mitochondrial membrane potential (Keij *et al.*, 2000) it was possible that the reduction in mitochondrial numbers per platelet was due not to their degradation within the lysosome but to an inability of the dye to stain depolarised mitochondria that remained within the platelet. To address this possibility the amount of cardiolipin (a mitochondria-specific phospholipid) left within viable platelets was assessed indirectly by incubating platelets with 10-*N*-nonyl acridine orange (NAO), a fluorescent dye that is used widely for the measurement of mitochondrial mass (Leprat *et al.*, 1990; Dumas *et al.*, 1995) and has also been used for the demonstration of mitochondrial loss by autophagy (Kirkland *et al.*, 2002) (fig. 5.7). Ageing platelets *in vitro* led to a decreased ability to accumulate NAO as assessed by cytometry (21.0 ± 1.2 mfu when fresh to 17.1 ± 0.8 mfu at 24 hours and 14.9 ± 1.6 mfu after 48 hours; $n=11$ donors, $p<0.01$) (fig 5.7.A). The decreased ability to accumulate NAO from day 0 to day 1 was 18.4% and tallies with the decrease in the number of mitochondria per platelet from approximately 9 to 7 (a 22% decrease) during the same period of incubation (fig 5.7.B). Thus it appeared that the changes in mitochondrial numbers observed with MTG are due to a real decrease in the average number of mitochondria per platelet and not due to changes in their membrane potential.

5.2.11 Vacuole formation is completely inhibited by MgSO_4 .

One of the caveats of maintaining platelets in a resting state during storage is the removal or reduction of the extracellular calcium concentration. It is known that under low calcium conditions the fibrinogen receptor / $\alpha_{\text{IIb}}\beta_3$ integrin complex can dissociate (Fujimura and Phillips, 1983; Zucker et al., 1983) and that the α_{IIb} subunit can homodimerise in trans leading to the zippering-up of the open canalicular system (Hanau et al., 1991; Gachet et al., 1992; Gachet et al., 1993). Although these reports relied on the ability of EDTA to chelate calcium and dissociate the integrin complex within a short time frame (generally less than 5 minutes) it was reasoned that, under the chronically low calcium conditions of incubation used in this thesis, there would also be a dissociation of the integrin complex. Furthermore, due to the zippering of the OCS, membrane fluidity and endocytosis would be affected, thus leading to an artefactual nutrient shortage that might provide the driving force of the lysosomal vacuole formation. To address this possibility the expression of the α_{IIb} subunit was examined and lysosomal vacuole formation was reassessed in platelets aged in the presence of 2mM magnesium, which can maintain the integrin complex.

During overnight storage of platelets as citrated PRP there was complete loss of α_{IIb} epitope recognised by the antibody as determined by microscopy and flow cytometry (fig. 5.8). Supplementation of the incubation medium with 2mM magnesium sulphate resulted, as expected, in complete retention of the α_{IIb} epitope. Further to this, there was complete inhibition of vacuole formation in the presence of MgSO_4 (fig. 5.9). It has already been noted that, in the absence of magnesium, vacuole formation was inhibited

by 3-MA but that this was also toxic to platelets; surprisingly when platelets were incubated in the presence of MgSO_4 the toxic effect of 3-MA was lost (fig. 5.9.B). The correlation between changes to α_{IIb} expression and vacuole formation prompted the examination of 3-MA's ability to alter α_{IIb} expression as a possible mechanism of its ability to inhibit vacuole formation. 3-MA had no effect upon α_{IIb} expression indicating that its ability to inhibit vacuole formation was independent of changes to the expression of α_{IIb} (fig. 5.9.C).

5.2.12 Total protein does not decrease during *in vitro* storage.

A cursory examination of total protein levels in platelets stored either in the presence or absence of exogenous amino acids did not reveal any differences in total protein as assayed by the Bradford method, nor were there any noticeable changes to the coomassie blue-stained protein profiles of stored platelets (fig 5.10).

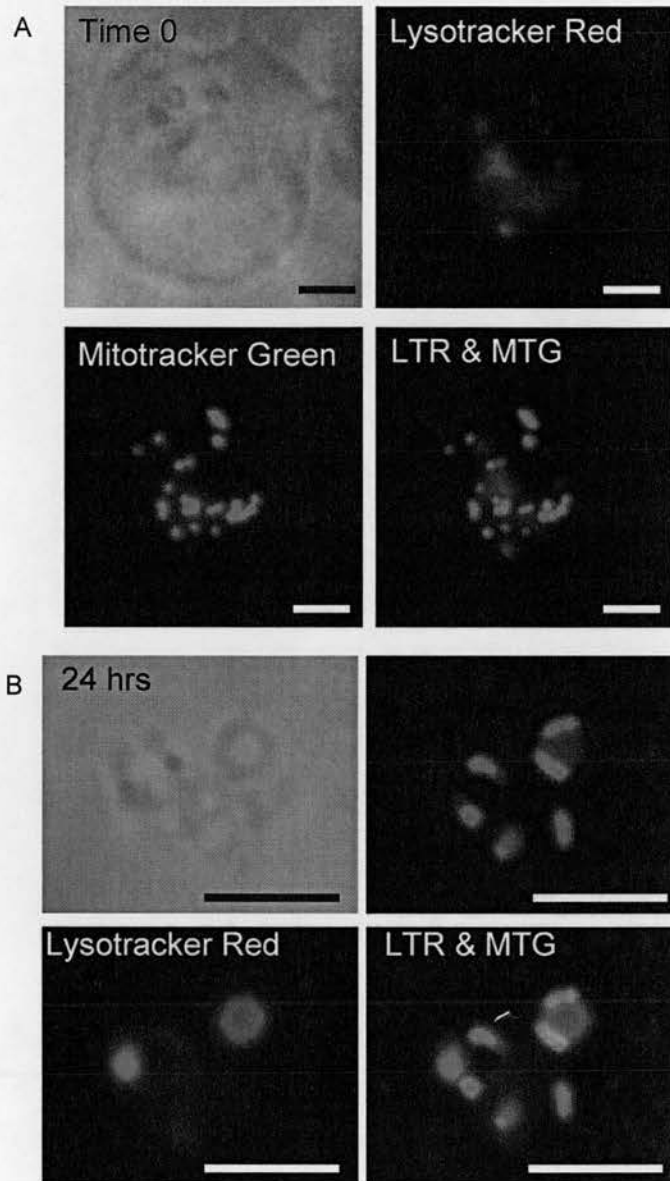


Figure 5.6. Mitochondria are discrete organelles within platelets and can be found in close association with vacuoles.

Fresh (A) or 24 hour-aged (B) platelets were incubated with mitotracker green and lysotracker red. Mitochondria are easily identified in spread platelets and are not associated with lysosomes. Incubation for 24 hours leads to the formation of vacuoles that can be seen to have associated with mitochondria. Bar = 2 μ M. Micrographs are representative of many platelets from several donors.

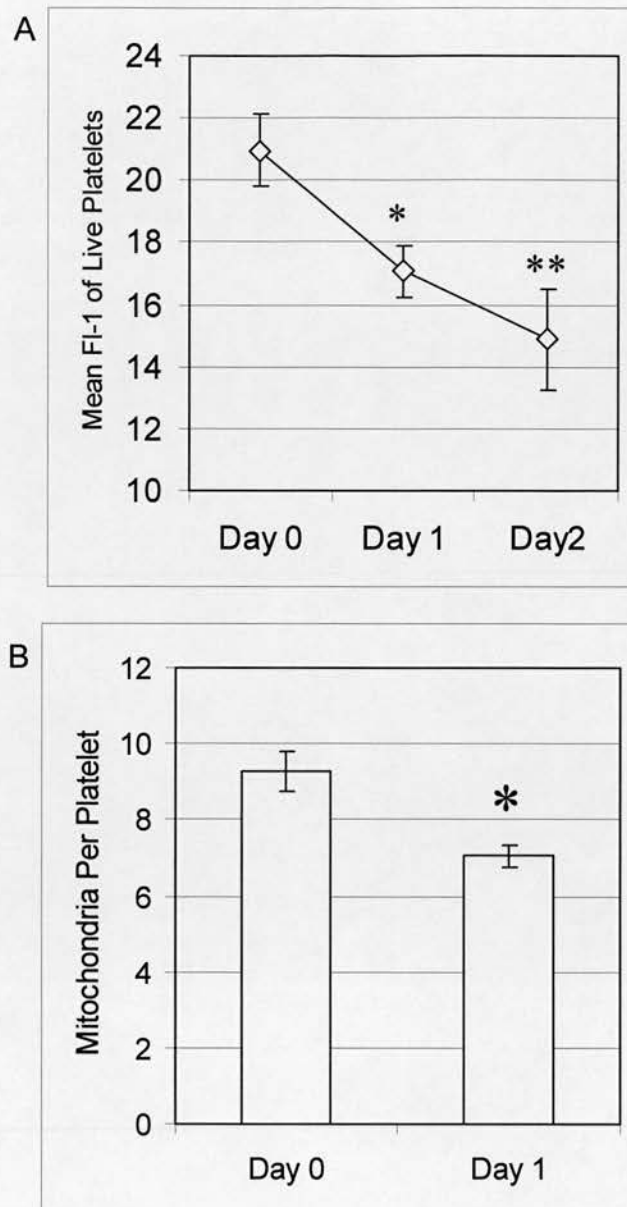


Figure 5.7. Mitochondrial signals are depleted during *in vitro* storage.

Mitochondria were quantified in spread platelets at time 0 and after 24 hours storage *in vitro*. Mitochondria were visualised with 100nM mitotracker green and at least 50 platelets per donor were examined (A); N=5; +/-SEM. Cardiolipin levels were determined using 500pM nonyl-acridine orange. NAO accumulates within viable platelets and fluoresces green. NAO shows reduced accumulation within platelets aged for 48 hours (B); n=13; +/-SEM; *P<0.01 vs previous time point; **P<0.05 from day 1.

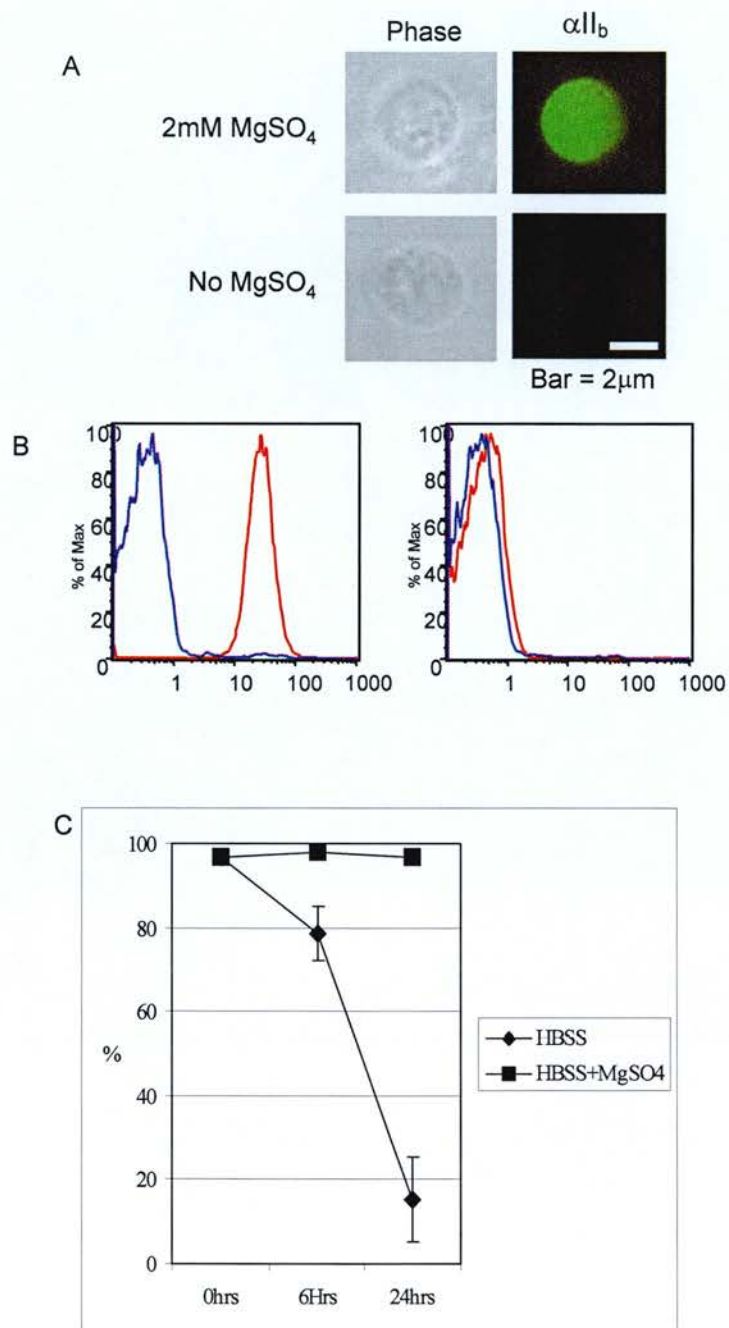


Figure 5.8. Loss of αIIb during *in vitro* storage.

Platelets were examined for the surface expression of αIIb during *in vitro* storage. Loss was confirmed by microscopy (A) and flow cytometry; blue line was autofluorescence set with irrelevant IgG and red was anti- αIIb (B) and could be blocked by supplementation of platelet incubates with 2mM MgSO_4 . Changes to the surface expression were examined at 6 and 24 hours (C); $n = 3$; \pm SE.

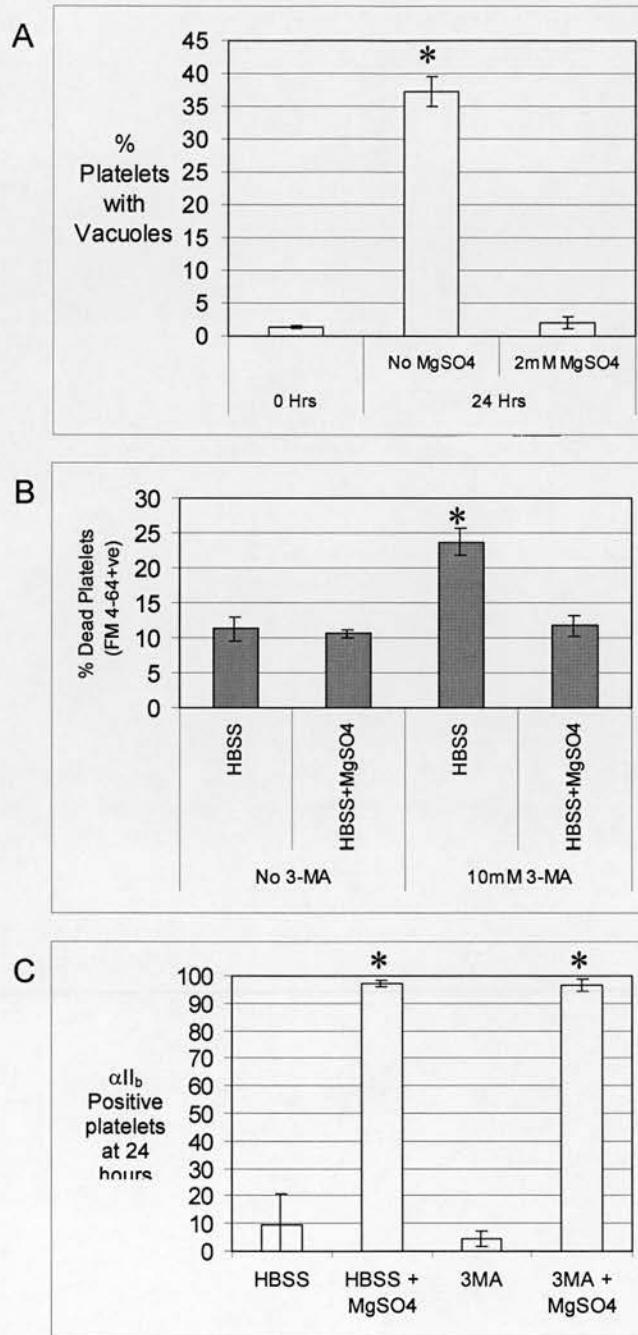


Figure 5.9. Vacuolisation is inhibited by magnesium sulphate and the toxicity of 3-MA is only realised when vacuolisation occurs.

Platelets were examined for the presence of Lysotracker red positive vacuoles when fresh and when aged over night in the absence or presence of MgSO₄ (A). Viability of platelets aged overnight in the presence or absence of 2mM MgSO₄ with and without 10mM 3-Methyadenine was examined by cytometry using FM4-64 as a vital stain (B). The effect of 3-MA on the surface expression of α II_b was examined after 24 hours of *in vitro* storage. ($n > 3$; * $P < 0.05$; +/-SEM)

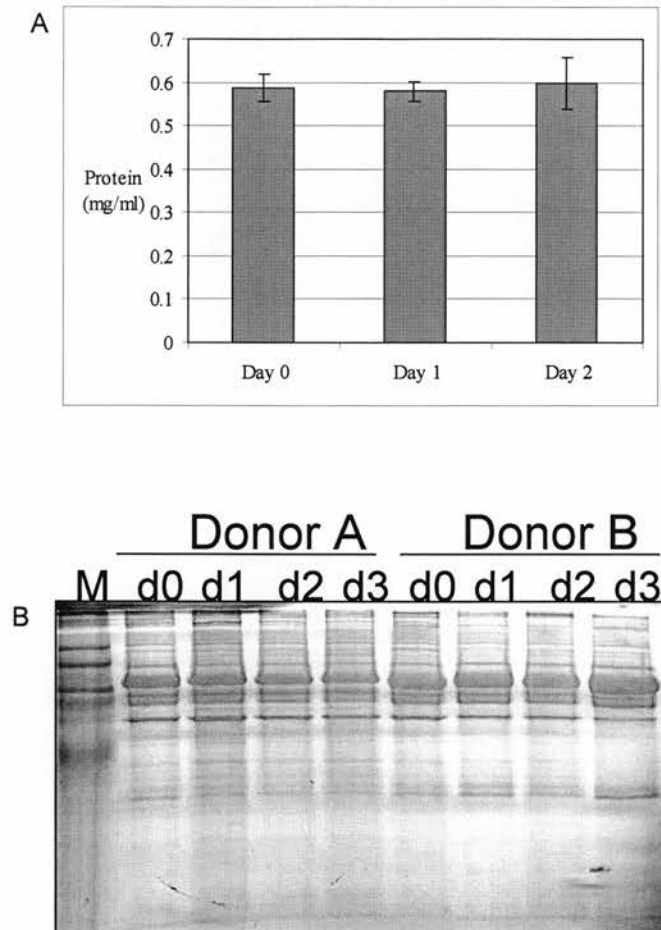


Figure 5.10. Total protein is not lost from platelet incubates nor is there gross signs of proteolysis associated with platelet pellets.

Platelet protein concentrations were estimated by the Bradford method from pellets collected by centrifugation ($n=4$; \pm SEM) (A). Protein profiles were obtained from platelets samples obtained after immediate isolation (d0) or after 24, 48 or 72 hours (d1, d2 and d3, respectively) incubation at 37°C. Platelet protein samples were electrophoresed through 6% polyacrylamide under reducing conditions profiles and polypeptides detected using coomassie brilliant blue (B).

5.3 Discussion.

The caspase-independent cell death of platelets described by some (Brown *et al.*, 2000) and eluded to by others (Bertino *et al.*, 2003; Wadhawan *et al.*, 2004) and the observation in this thesis of a necrotic-like end point for stored platelets led to the hypothesis that their death was autophagic in nature. Autophagic cell death is persistently associated with vacuolisation and destruction of cytoplasmic contents including organelles leading to near complete catabolism and a necrotic-like end point (Thummel, 2001; Lee and Baehrecke, 2001; Bera *et al.*, 2003). This contrasts the phenotype associated with apoptosis; preservation of organelles and maintenance of plasma membrane integrity (Kerr *et al.*, 1972). However, it is important to note that there appears to be overlap between the mechanisms of autophagic and apoptotic cell death (Bursch *et al.*, 2000; Uchiyama, 2001; Lee *et al.*, 2003).

The first step in the establishment of autophagy in platelets was an examination of internal membrane morphology at the electron microscope level. Autophagic sequestration of cytosol and organelles involves the formation of a pre-autophagosomal (Suzuki *et al.*, 2001) structure followed by the *de novo* synthesis of a double-membrane limiting vesicle, termed the autophagic vacuole. This vacuole in mammalian cells has a characteristic multi-lamellar morphology that is readily identifiable at the TEM level (Eskelinen *et al.*, 2002). Examination of either freshly isolated or overnight aged platelets at the TEM level never revealed the existence of any multi-lamellar structures

that could be identified as autophagic vacuoles. In stored but not fresh platelets the presence of large vacuoles was observed but, except for the deposition of glycogen around their perimeter, there were no signs of identifiable organelles within these structures. It is likely therefore, that macro-autophagy is not the cause of the large vacuoles.

Accumulation of the dyes lysotracker red and monodansylcadaverine to large vacuole structures was consistent with an autophagic phenotype (see appendix fig. 8.5) however the fact that the vacuoles *and* lysosomes in fresh platelets accumulated MDC indicates that this dye is not suitable for the specific detection of autophagic vacuoles, a conclusion reached by others (Elmore *et al.*, 2001) and Aviva Tolkovsky (*pers. comm.*). Confirmation that the vacuoles were indeed of a lysosomal origin was obtained using the antibodies to the lysosome specific protein, Lamp2.

Changes to the amount of MDC accumulated by platelets within a 20 minute period with the dye did not alter after *in vitro* incubation, indicating that the vacuoles were not synthesised *de novo* but that they had formed from the original lysosomal compartment.

The observation that the average number of mitochondria in platelets decreases during storage and that this tallied with a reduction in the amount of NAO that associated with viable platelets suggested that the vacuole formation may have been the result of lysosomal fusion with, and destruction of, effete / damaged mitochondria. Mitochondrial disappearance from cells occurs in response to nerve growth factor

(NGF)-starvation in primary neuron cultures (Xue et al., 1999; Tolkovsky et al., 2002) and serum starved hepatocytes (Elmore *et al.*, 2001). This disappearance reflects an ability of the cell to selectively destroy mitochondria in the lysosome indicating that autophagy can be a highly selective process. Examination by fluorescence microscopy of platelets loaded with MTG and LTR revealed on rare occasions that mitochondria had become clustered around the large vacuoles, however co-localisation of the dyes was never observed. It is reasonable to think that if mitochondria were fusing with lysosomes to form large vacuoles, at some point co-localisation between MTG and LTR should have been observed. Therefore the apparent disappearance of mitochondria is not likely the result of crinophagic fusion with the lysosomes. In support of this conclusion is an observation that serum starved, glucagon treated rat hepatocytes up-regulated the number of LTR-labelled acidic compartments and that mitochondria without a membrane potential (measured by the sudden green fluorescence caused by the loss of quenching that occurs when mitochondria co-loaded with MTG and Tetramethylrhodamine methylester (TMRM) lose $\Delta\Psi_m$ and leak TMRM) were sequestered into these acidic structures, a result that could be blocked by maintaining $\Delta\Psi_m$ with cyclosporin A which is able to block the permeability transition pore (Elmore *et al.*, 2001).

Another explanation for the reduction in the average number of mitochondria per platelet and the reduced NAO fluorescence is the possibility that the platelets with the largest number of mitochondria die first.

Vacuole formation is sensitive to 3-MA, a drug extensively used in the field of autophagy research. However, used at 10mM it has non-specific effects on cells such as an ability to increase cAMP (Caro *et al.*, 1988) and can inhibit endosomal transport to lysosomes (Punnonen *et al.*, 1994) - yet many groups have based the identification of an autophagic mechanism solely on the effects of 3-MA (Sandvig and van Deurs, 1992). In the present study 3-MA was able to incompletely block vacuole formation during *in vitro* storage. This inhibition was accompanied by toxicity. This may indicate that vacuole formation, if autophagic, was a survival strategy invoked by *in vitro* conditions whose inhibition would promote platelet death. This is supported by the observation that 3-MA was only toxic to platelets when vacuolisation was occurring; toxicity was lost in the presence of MgSO₄, which was able to completely block vacuolisation. However, a complicating factor in this conclusion is the observation that 3-MA was able to completely inhibit platelet activation in response to various agonists, indicative of non-specific effects of the drug on platelet biology. This ability to inhibit platelet activation appeared to not just affect degranulation and integrin activation but also very early events linked to shape change. The fact that activation in platelets is inhibited at the shape-change stage is suggestive of increased intracellular cAMP concentration, which is known to occur in isolated hepatocytes treated with 3-MA (Caro *et al.*, 1988). Thus it is not clear if the toxicity associated with 3-MA is linked to its ability to block vacuole formation or whether it is the result of an effect unrelated to autophagy that is only realised in the absence of magnesium. Although untested, if levels of cAMP are constitutively high in the platelets this may represent a drain on cellular ATP that leads to loss of viability. Therefore the results with 3-MA may be due to indirect toxic effects on platelets. However, the fact that 3-MA was capable of completely inhibiting platelet

activation is further evidence that platelet death in this model can occur independently of platelet activation.

Amino acid starvation is used to experimentally stimulate autophagy in a wide variety of cells (Lenk et al., 1999; Mordier et al., 2000) therefore it was possible that vacuole formation was a response to one or a number of amino acids becoming limiting. Supplementation of platelet incubates with amino acids was able to partially reduce vacuole formation in platelets but had no effect on platelet survival, contrasting the results obtained with 3-MA; this suggests that vacuole formation and platelet survival were independent of each other. However, this conclusion is tentative due to the known divergence of autophagic responses to starvation (Schworer and Mortimore, 1979). Autophagic responses elicited by glucagon involve lysosomal glycogen breakdown whereas those elicited by amino acid starvation lead to proteolysis. However, amino acid supplementation can reduce glycogen autophagy in glucagon treated hepatocytes (Schworer and Mortimore, 1979). Therefore vacuolisation may be a response to carbohydrate limitation and that amino acid supplementation, although able to down-regulate vacuole formation, is not sufficient to provide a pro-survival signal and compensate for the carbohydrate limitation.

Examination of platelet function in response to convulxin revealed that amino acids could retain better platelet function than non-supplemented platelets. This suggests that loss of platelet function is linked to increasing vacuolisation and may suggest that amino acid uptake by platelets is key not for survival but for maintenance of functional responsiveness to agonists. The fusion of storage granules with the lysosome is known

as crinophagy but the process has never been described in platelets. It is tempting to think that the collection of observations made in this work is consistent with a crinophagic process of granule destruction. This autophagic response may have been initiated by a stress linked to low cations, such as OCS zippering, but one that could be obviated by magnesium. Although not having any effect on viability it has profound effects on platelet function.

Although total protein levels did not decrease during *in vitro* storage further work should examine more specifically the elements of degranulation that appear to be protected by amino acid supplementation. An examination of the amount of alpha granule protein retained by platelets supplemented with amino acids relative to controls and an examination of the contents of vacuoles would be beneficial in understanding the role of vacuolisation. Indeed, the lack of total protein changes during storage may indicate that dense granules rather than alpha granules are fusing with lysosomes; consistent with the amino acid data this would reduce the amount of releasable serotonin, calcium and ADP and negatively affect CD62P expression during degranulation.

Also, the presence of LC-3 in platelets should be confirmed and, if present, its sub-cellular localisation examined. Efforts to establish the presence of LC-3 were unsuccessful due to technical difficulties in optimising western blotting conditions and the highly limited availability of this non-commercial antibody (a very kind gift of Takahashi Ueno).

This work dealt with the hypothesis that autophagy was involved in the caspase independent cell death of platelets during *in vitro* incubation. The large vacuoles that formed *in vitro* were identified as lysosomal in origin but it is not clear why they have become enlarged. A single factor leading to vacuolisation was not identified in this work and it remains obscure as to why vacuolisation occurs when platelets are held in citrated plasma. The current data support the hypothesis that the enlargement of lysosomes in platelets is the result of crinophagy, a fusion of granules with the lysosome that is controlled by divalent cation availability. In conclusion it is unlikely that macroautophagy is responsible for platelet death *in vitro*, however the possibility remains that vacuolisation is an autophagic response to changes in conditions important for the maintenance of platelet function.

The lack of a change to total platelet protein indicates that although necrosis may have been detected at the TEM level (and possibly by the platelets' inability to accumulate calcein-AM) that no substantial leakage of polypeptides to the extracellular milieu occurs. This contrasts the known result of necrosis, which is typified by rapid and irreversible loss of cellular integrity with consequent pro-inflammatory spillage of cellular contents. Therefore, the retention of protein within dying platelets argues for a more elaborate form of cell death than necrosis.

6 GLYCOGEN INFLUENCES PLATELET SURVIVAL.

6.1 Abstract.

Platelets possess large fields of glycogen and express few GLUT transporters on their surface whilst in their resting state. This led to the hypothesis that glycogen and / or an ability to catabolise glycogen is a determinant of the platelet's lifespan *in vitro*. It was confirmed at both the light and electron microscopic level that platelets contain large deposits of glycogen. These glycogen deposits, as measured by the anthrone assay, become depleted from platelets during incubation at 37°C. The depletion of glycogen from platelets could be partially inhibited by the glycogen phosphorylase inhibitors, caffeine and the novel caffeine analogue, CP-91149. Lithium was also capable of partially inhibiting glycogen depletion from platelets, possibly by indirectly antagonising glycogen phosphorylase activity. All three agents were also toxic to platelets as assessed by the calcein-AM accumulation assay providing evidence of a correlation between an ability to breakdown glycogen and maintenance of platelet viability. Interestingly, hypoglycaemia caused changes to the platelets' ability to accumulate NAO and calcium_(i), suggesting that low exogenous glucose promotes fundamental changes to mitochondria and calcium homeostasis but that these changes do not translate to toxicity. Consistent with this hypothesis were the observations that platelets maintained with antimycin did not die and that deliberate collapse of mitochondrial membrane potential did not promote platelet death either. Hypoglycaemia appeared to prime platelets to the cell death promoting ability of antimycin and BH3I-2', a small molecule promoter of pro-apoptotic Bcl-2 family balance within cells.

6.2 Results.

6.2.1 Platelets possess large fields of glycogen.

Platelets examined at the light microscope level after PAS staining contained abundant PAS-positive material (fig. 6.1) consistent with the size of glycogen fields. By TEM glycogen could be seen in close proximity of a membrane limiting structure presumed to be part of the open canalicular system (fig. 6.2). Although not shown, it was clear from examination of electron micrographs of platelets that had been incubated at 37°C for 72 hours that platelets contained very little glycogen.

6.2.2 Glycogen content of platelets is dramatically reduced during storage.

To explore quantitatively the glycogen content of platelets the anthrone assay was employed. The anthrone assay estimates the amount of glucose hydrolysed from total carbohydrate isolated from cells by alkaline precipitation, thus results describe glucose concentrations rather than glycogen content. Glycogen content of platelets was estimated from a pellet of platelets harvested from 1ml of autologous PRP. Results are therefore expressed as micrograms of glucose per ml of platelets.

Freshly isolated platelets yielded 44.8 \pm 6.2 μ g glucose/ml platelets, whereas by 24 hours the content of glycogen had fallen to 18.4 \pm 1.8 μ g glucose/ml platelets and by 48 and 72 hours to 12.0 \pm 1.3 and 5.3 \pm 2.8 μ g glucose/ml platelets, respectively ($n > 3$;

$P < 0.01$) (fig. 6.3). This data are consistent with the observation made at the TEM level that platelets lose glycogen during *in vitro* incubation.

6.2.3 Hypoglycaemic conditions do not affect the initial rate of glycogen loss from platelets.

Using the anthrone assay it was established that platelets held under hypoglycaemic conditions for 24 hours did not lose a significantly greater amount of glycogen when compared to control platelets (47.2 \pm 5.0 μ g glucose/ml platelets when fresh; 14.7 \pm 6.6 μ g glucose/ml platelets after 24 hours and 12.7 \pm 3.7 μ g glucose/ml platelets after 24 hours without exogenous glucose; $n = 3$; $P < 0.01$ between fresh and incubated platelets; $P = 0.4$ between control and hypoglycaemic platelet samples) (fig. 6.4). Thus, hypoglycaemia did not promote the loss of glycogen from platelets, consistent with the hypothesis that glycogen was the main source of glucose for metabolism in platelets during their *in vitro* incubation.

6.2.4 Three distinct pharmacological agents partially inhibit glycogen depletion in platelets.

If glycogen is an important source of metabolic glucose it was reasoned that inhibition of its catabolism would have negative effects on platelet viability. In order to test this

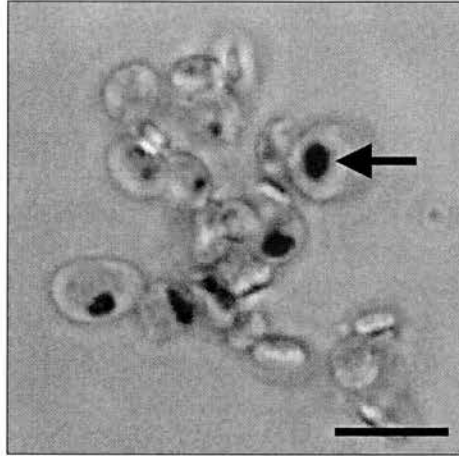


Figure 6.1. Micrograph of fixed platelets stained with periodic acid – Schiff reagent.

Platelets were aged for 24 hours, fixed with 1 volume of 2% formaldehyde and stained using PAS reagent. PAS positive material can be readily identified within platelets (arrow); these areas correspond to the expected size of glycogen fields as seen by transmission electron microscopy. Bar = 5 μ m.

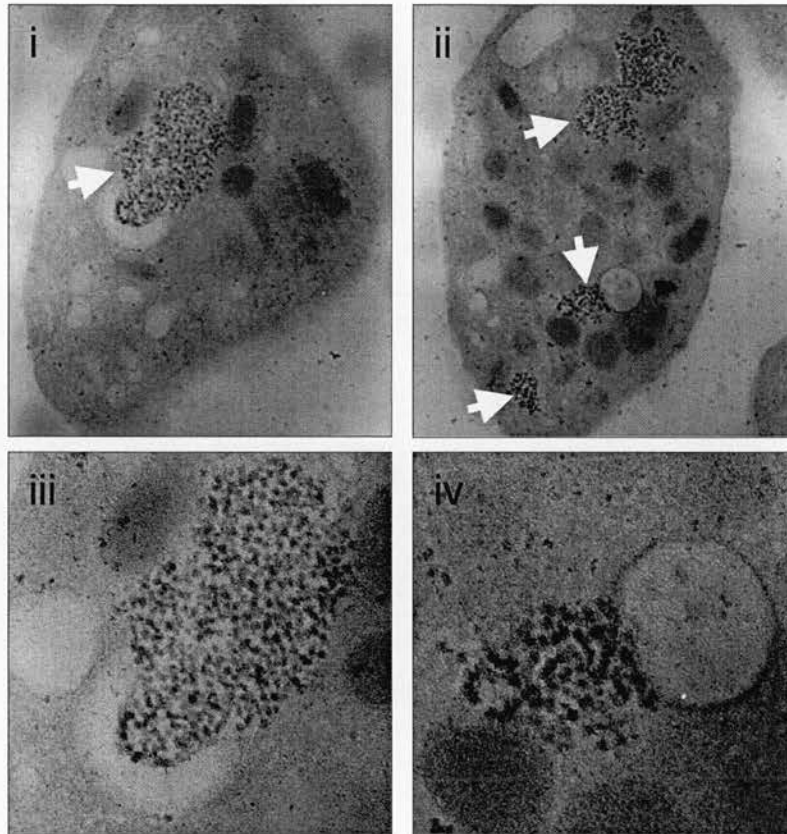


Figure 6.2. Glycogen can be seen associated with the OCS within freshly isolated platelets.

Freshly isolated platelets were fixed for 1 hour at 37°C with 3% glutaraldehyde and then processed for transmission electron microscopy. Micrographs show two platelets from the same donor containing large fields of glycogen (arrows), some of which is closely associated with what appears to be the open canalicular system. Micrographs iii and iv are close-ups of i and ii, respectively.

hypothesis it was necessary to find inhibitors of glycogen phosphorylase, the major regulatory enzyme of glycogen catabolism. Caffeine and the novel caffeine analogue, CP-91149, are known to be direct inhibitors of glycogen phosphorylase (Martin *et al.*, 1998), however they are thought to act at distinct sites on the enzyme. An alternative inhibitor, Lithium, reduces glycogen catabolism by blocking the phosphorylation of glycogen synthase thereby shifting the equilibrium of glycogen metabolism towards synthesis rather than catabolism. Glycogen synthase and phosphorylase activities are tightly coupled and it is thought that inhibition of one results in the promotion of the other, therefore lithium would be expected to exert promotion of synthase activity whilst simultaneously inhibiting phosphorylase activity (Latsis *et al.*, 2002).

Incubation of platelets for 24 hours at 37°C in the presence of lithium chloride (20mM), caffeine (20mM) or CP-91149 (100µM) incompletely inhibited the loss glycogen (freshly isolated platelets contained 45.7 +/-1.5µg glucose/ml platelets; whereas platelets incubated *in vitro* for 24 hours contained 23.2 +/-2.2µg glucose/ml platelets; whereas platelets incubated in the presence of lithium or caffeine contained 31.7 +/-2.2 and 30.5 +/-0.2µg glucose/ml platelets, respectively; $n>3$; $P<0.01$ between fresh and incubated platelets; $P<0.05$ between control and lithium or caffeine supplemented platelet incubates) (fig.6.5). This data indicates that glycogen breakdown is sensitive, in part, to inhibition of phosphorylase by the direct action of caffeine or CP-91149 or by the indirect action of lithium and support the contention that loss of glycogen from platelets is due to a metabolic process involving phosphorylase activity.

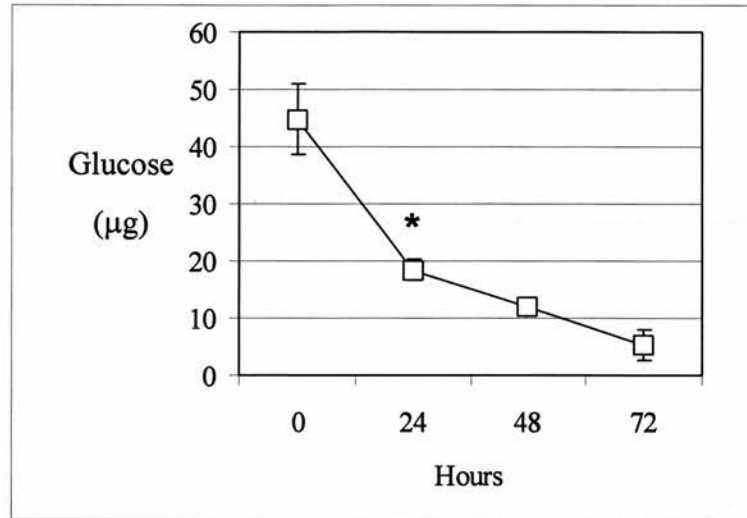


Figure 6.3. Glycogen is lost from platelets during *in vitro* storage. Platelets were aged as PRP for 72 hours. Loss of glycogen was estimated as the reduction in total platelet carbohydrate by the anthrone assay against glucose standards of known concentration. * $P < 0.01$; $n = 4$; \pm SEM.

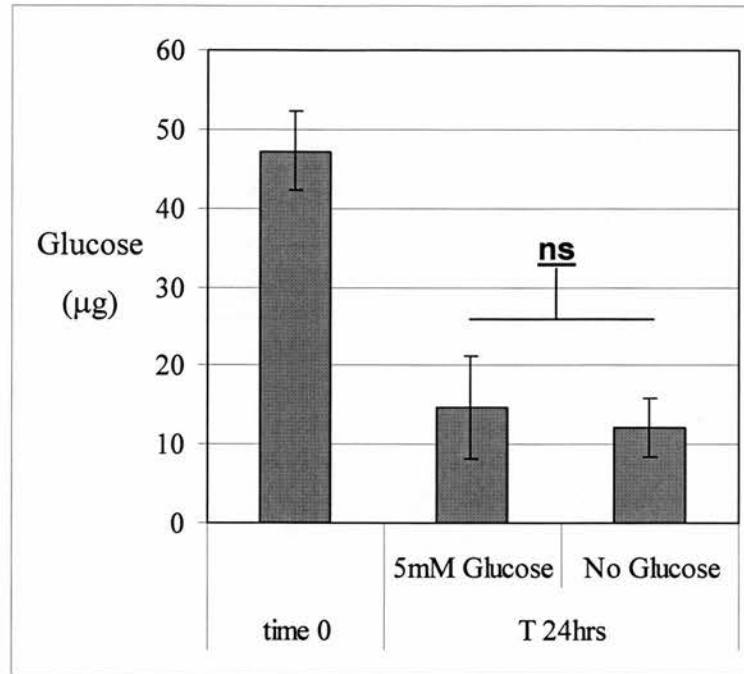


Figure 6.4. Hypoglycaemic conditions do not affect the rate of loss of Glycogen from platelets.

Platelets were incubated for 24 hours as a 1:50 dilution of PRP supplemented with or without 5mM glucose. No significant difference in the amount of glycogen remaining associated with platelets at 24 hours was detected. $P=0.44$; $n = 4$; \pm -SEM.

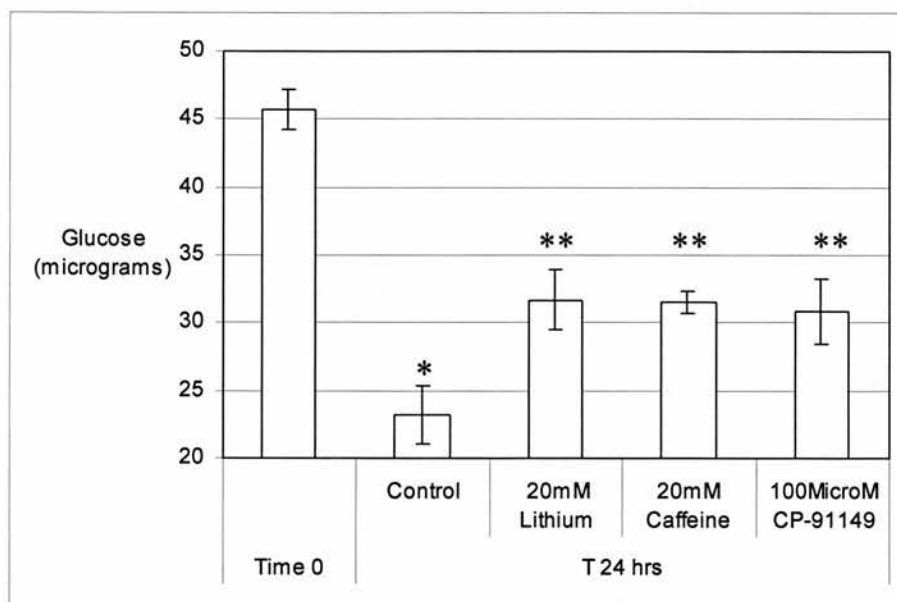


Figure 6.5. Lithium, Caffeine and CP-91149 inhibit the loss of glycogen from platelets during *in vitro* incubation.

Platelets were incubated for 24 hours as PRP supplemented with 20mM LiCl₃, 20mM caffeine or 100μM CP-91149. All three agents caused an inhibition of glycogen loss from platelets. *P<0.01 from time 0; **P<0.05 from 24 hour control samples; *n* = 4; +/-SEM.

6.2.5 Lithium, caffeine and CP-91149 promote platelet death.

The next question was whether or not inhibition of glycogen catabolism correlated with increased platelet death. To explore this hypothesis, platelets were incubated under control or hypoglycaemic conditions with lithium or caffeine and assessed 72 hours later for viability using the calcein accumulation assay.

Lithium promoted the loss of viability from platelets within 72 hours of incubation (43.15 \pm 4.4 vs 56.7 \pm 2.4% dead under normoglycaemic without or with lithium and 48.2 \pm 7.4 vs 72 \pm 2.6% dead under hypoglycaemic conditions without or with lithium; $P < 0.05$ for each difference) (fig. 6.6.A). Incubation of platelets with caffeine gave similar results (32.9 \pm 3.2 vs 44.7 \pm 5.5% dead under normoglycaemic without or with caffeine and 37.3 \pm 3.0 vs 50.1 \pm 4.2% dead under hypoglycaemic conditions without or with caffeine; $P < 0.05$ for each difference) (fig. 6.6.B).

In a similar experiment platelets were incubated with the novel caffeine analogue CP-91149 (100 μ M) for 24 hours (preliminary data indicated that by 72 hours most platelets were dead) and examined for viability using the calcein accumulation assay (fig. 6.7). It was found that this agent also had a profound effect upon platelet survival *in vitro*. Platelet death at 24 hours was significantly promoted in the presence of CP-91149 (11.4 \pm 1.3% versus 23.8 \pm 2.4% dead without or with CP-91149, respectively; $P < 0.05$; $n = 3$) and its effects, like that of caffeine and lithium were further promoted by hypoglycaemia (12.0 \pm 1.6% versus 37.6 \pm 4.5% dead without or with CP-91149,

respectively; $P < 0.05$; $n = 3$). Thus, three agents having broad pharmacological targets but sharing an ability to inhibit glycogen phosphorylase are able to promote platelet death *in vitro*.

6.2.6 Hypoglycaemia alters mitochondrial integrity.

The data so far are consistent with glycogen content or an ability to utilise glycogen as being an important determinant of platelet lifespan *in vitro*. However an apparent contradiction had arisen regarding the importance of exogenous glucose to platelet survival; platelets held under hypoglycaemic conditions did not deplete their glycogen stocks more rapidly than controls but were more sensitive to the effects of caffeine, CP-91149 and lithium. Furthermore, hypoglycaemia had no effect upon platelet viability as assessed by the calcein-AM accumulation assay (figs. 6.6 and 6.7), even over a five day period of incubation ($n > 3$; $P=0.99$) (fig 6.8). However, it was noted that a discrepancy had arisen between the calcein-AM and the NAO-accumulation assays. Failure to accumulate calcein was observed in $8.4 \pm 0.8\%$ and $9.3 \pm 0.6\%$ of platelets held for 24 hours under normo- and hypoglycaemic conditions, respectively ($n=5$; $P=0.08$); whereas $12.8 \pm 1.2\%$ and $22.2 \pm 1.8\%$ of platelets failed to accumulate NAO after 24 hours incubation under normo- and hypoglycaemic conditions, respectively ($n=5$; $P<0.05$) (fig. 6.8). This result indicated that hypoglycaemia had affected the integrity of platelet mitochondria but that this change had not translated into a loss of viability.

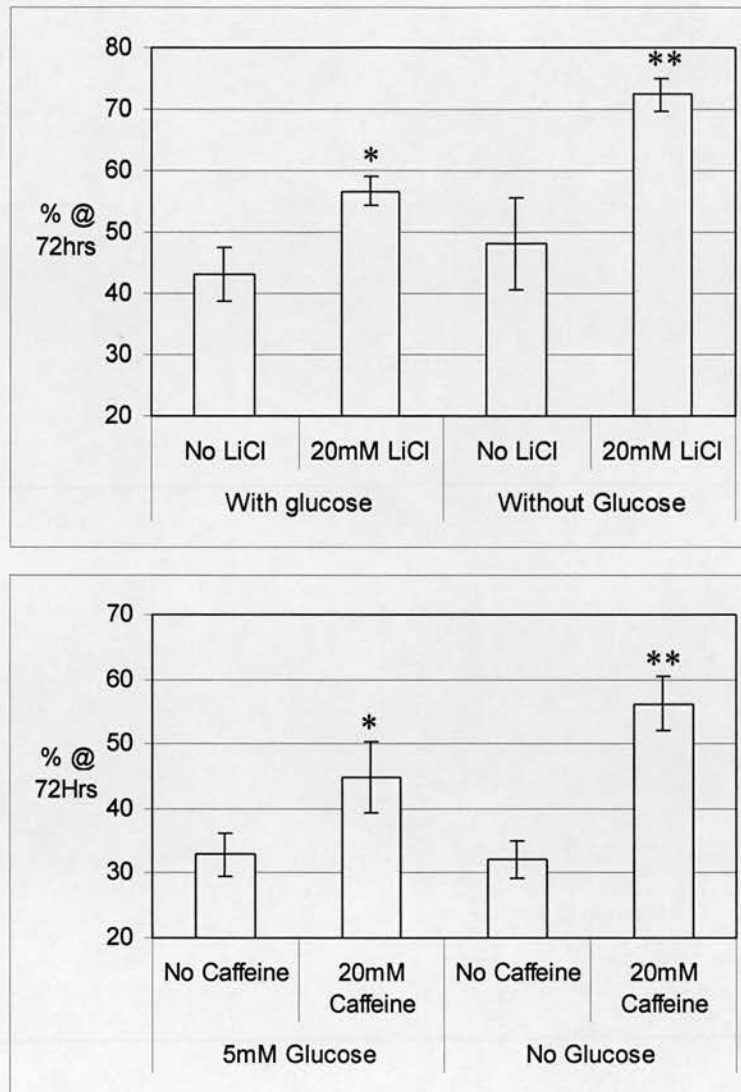


Figure 6.6. The effect of lithium and caffeine on platelet viability *in vitro*.

Platelets were incubated for 72 hours at 37°C as 1:50 dilution of PRP supplemented with either 5mM or no glucose. Lithium was added to a final concentration of 20mM. Viability was assessed by the platelets ability to accumulate calcein-AM. Lithium negatively affected platelet survival and this effect was exacerbated by hypoglycaemia. $n = 3$; \pm SEM * $P < 0.05$ from normoglycaemic control; ** $P < 0.05$ from hypoglycaemic control.

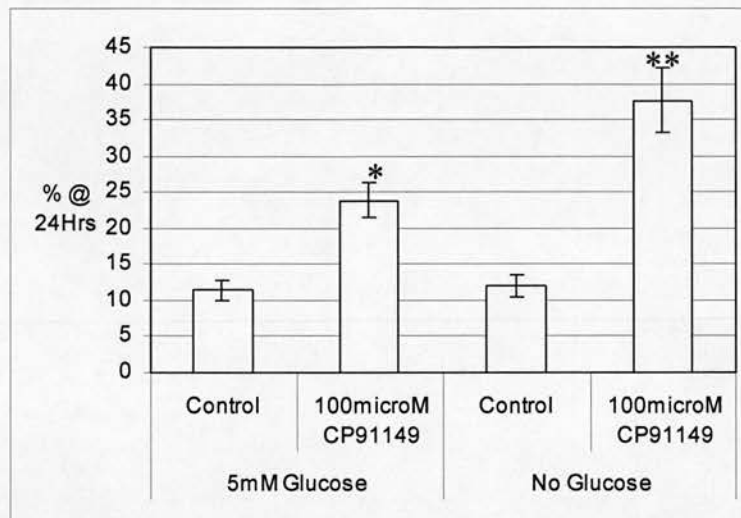


Figure 6.7. The effect on platelet viability *in vitro* of CP-91149 in conjunction with hypoglycaemia.

Platelets were incubated for 24 hours at 37°C as 1:50 dilution of PRP supplemented with either 5mM or no glucose. CP-91149 was added to a final concentration of 100µM. Viability was assessed by the platelets ability to accumulate calcein-AM or FM4-64. CP-91149 negatively affected platelet survival and this effect was exacerbated by hypoglycaemia. $n = 3$; \pm SE; * $P < 0.05$ from controls; ** $P < 0.05$ from normoglycaemic platelets supplemented with CP 91149.

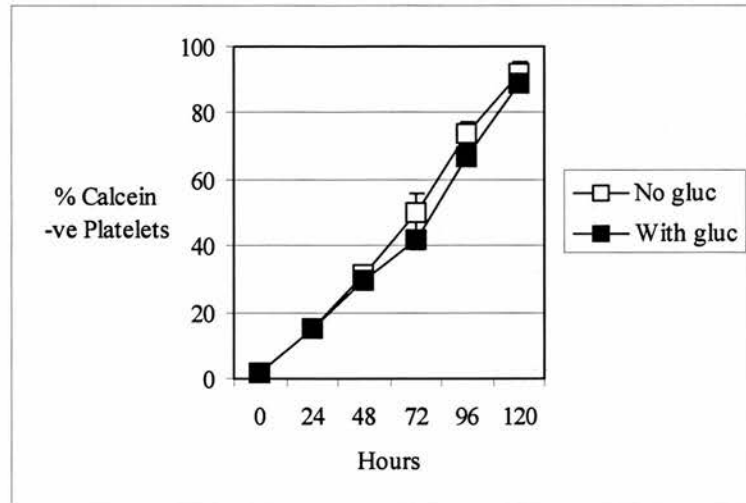


Figure 6.8. Hypoglycaemia does not affect platelet lifespan *in vitro*. Platelets were aged for 5 days as a 50-fold dilution of PRP supplemented with or without glucose to a final concentration of 5mM. Platelet viability was assessed using calcein accumulation assay. No differences between treatment groups in terms of platelet viability were observed over the 5-day period. This result suggests that platelets are not obliged to utilise exogenous glucose for vital processes. $n = 5$; $P=0.988$.

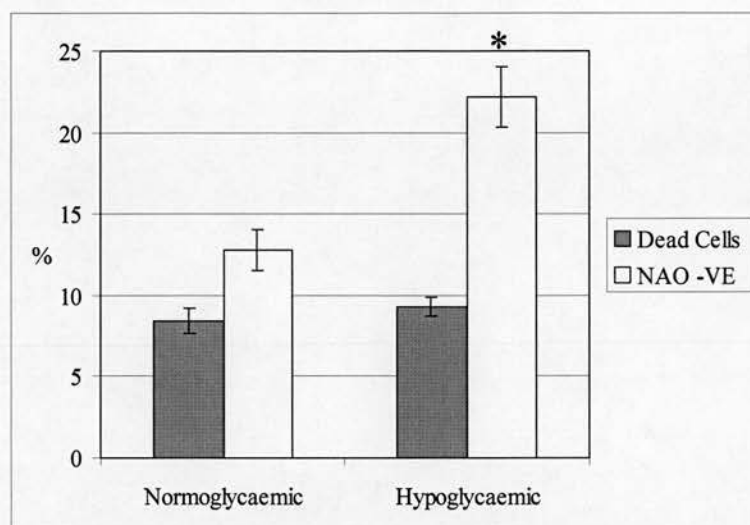


Figure 6.9. Hypoglycaemia alters platelets' ability to accumulate NAO but not Calcein-AM.

Platelets were aged for 24 hours *in vitro* at 37°C as 50-fold dilution of PRP supplemented either with or without 5mM glucose and assessed for viability using NAO or Calcein accumulation assays. As expected, no loss of viability in response to hypoglycaemia was detected with the calcein assay (shaded columns), however, the ability to accumulate NAO was affected by hypoglycaemia. n = 5; *P<0.05 compared to NAO^{-VE}s under normglycaemic conditions.

6.2.7 Changes to mitochondrial membrane potential are uncoupled from platelet death.

The data suggested that hypoglycaemia could promote changes to platelet mitochondria but that these changes do not necessarily translate into loss of viability. To explore this possibility platelets were incubated for 5 days and their mitochondrial membrane potential was assessed using the mitochondrial membrane potentiometric dye JC-1. Platelet death as assessed by calcein-AM accumulation proceeded at a linear rate over 5 days from less than 1.7 \pm 0.5% dead in fresh samples to 15.3 \pm 1.7%; 29.8 \pm 3.0%; 41.8 \pm 2.7%; 66.81 \pm 2.4% and 88.9 \pm 1.5% death on days one, two, three, four and five of incubation, respectively ($n = 5$; $P < 0.01$). This near linear rate of platelet death was contrasted by the data obtained with JC-1 which indicated that 9.3 \pm 1.4% of fresh platelets had lost their mitochondrial membrane potential and that this progressed as a hyperbolic kinetic to 37.8 \pm 2.6%; 54.6 \pm 3.6%; 69.6 \pm 4.3%; 82.8 \pm 2.3% and 88.9 \pm 1.5% of platelets on days one, two, three, four and five of incubation respectively ($n = 5$; $P < 0.01$) (fig. 6.10). This divergence of the kinetics regarding loss of $\Delta\Psi_m$ and viability as assessed by the JC-1 and calcein-AM accumulation assays, respectively, support the contention that changes to platelet mitochondria are uncoupled from loss of platelet viability.

Evidence for this conclusion was sought by incubation of platelets with an inhibitor of electron transport (antimycin) and a protonophore that causes loss of $\Delta\Psi_m$, carbonyl cyanide *m*-chlorophenylhydrazone (mCCCP). The rationale for this approach was that if mitochondrial function was unimportant for maintenance of platelet viability then these

two agents would not affect the frequency of platelets able to accumulate calcein-AM. Incubation of platelets with either antimycin A (10 μ g/ml) or mCCCP (10 μ M) for 24 hours at 37°C did not promote platelet death relative to diluent controls (16.3 \pm 2.5% control; 15.7 \pm 2.9% with Antimycin; n = 4; P =0.35 and 14.0 \pm 1.7% for control; 13.3 \pm 1.3% mCCCP; n = 3; P = 0.3) (fig. 6.11). Confirmation of the ability of antimycin to cause inhibition of electron transport in platelets (McElroy et al., 1971; Peerschke, 1999) was confirmed by its ability to reduce fibrinogen binding by 29% (9.2 \pm 1.8mfu and 3.9 \pm 0.5mfu for platelets activated in the presence or absence of antimycin, respectively) and CD62P expression by 57% (12.8 \pm 3.5mfu for the control and 9.1 \pm 2.4mfu for antimycin treated platelets; n = 4; P <0.05; for both fibrinogen binding and degranulation) in response to 5 μ M ADP (fig. 6.12). The collapse of $\Delta\Psi_m$ caused by mCCCP was reported by JC-1 (49.8 \pm 4.5% of platelets had an intact $\Delta\Psi_m$ at 24 hours whereas only 35.9 \pm 2.1% had an intact $\Delta\Psi_m$ when incubated in the presence of mCCCP (n = 3; P <0.01) (fig. 6.13).

6.2.8 Antimycin combines with hypoglycaemia to bring about platelet death.

The data supported the hypothesis that although mitochondrial integrity or function can be negatively affected by pharmacological agents there is no subsequent promotion of platelet death. Time course experiments also supported this contention, inasmuch that the kinetic of the platelets' loss of $\Delta\Psi_m$ is different from (and therefore uncoupled from) loss of viability. It was surprising then to find that incubation of platelets with 10mM 2-deoxy D-glucose (2DG) (2DG was used to effect hypoglycaemia in full cPRP,

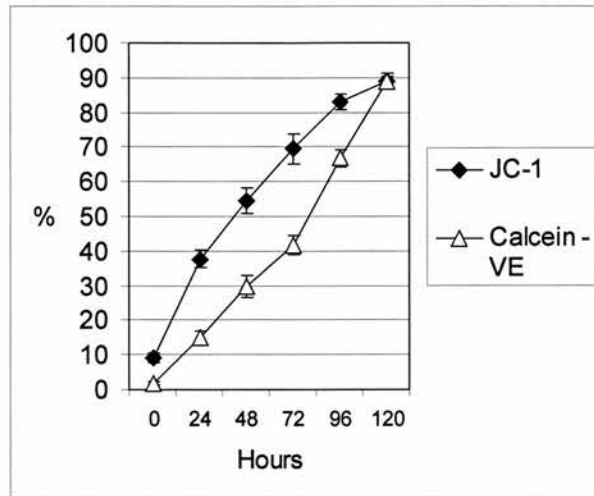


Figure 6.10. Platelet death during storage at 37°C is uncoupled from degranulation and changes to mitochondria.

Platelets were aged for 5 days as PRP and examined for surface expression of CD62P (A), loss of $\Delta\Psi_m$ using JC-1 (B) and retention of viability as assessed by an ability to accumulate calcein (Both graphs). $n > 5$ different donors for each parameter examined.

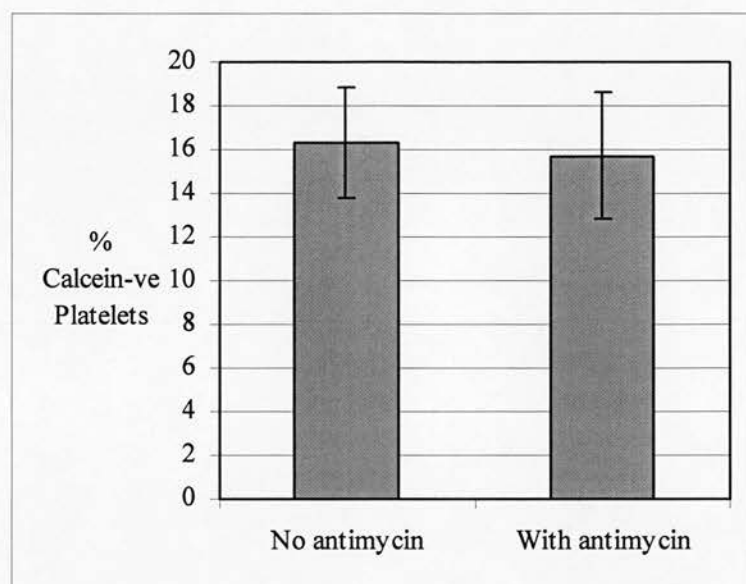


Figure 6.11. Antimycin A has no effect upon platelet viability.

Platelets were aged for 24 hours in the presence of 10 μ g/ml Antimycin A and examined for viability using the calcein accumulation. No significant difference in platelet viability was detected by 24 hours. $n=3$; $P=0.3$; \pm SEM.

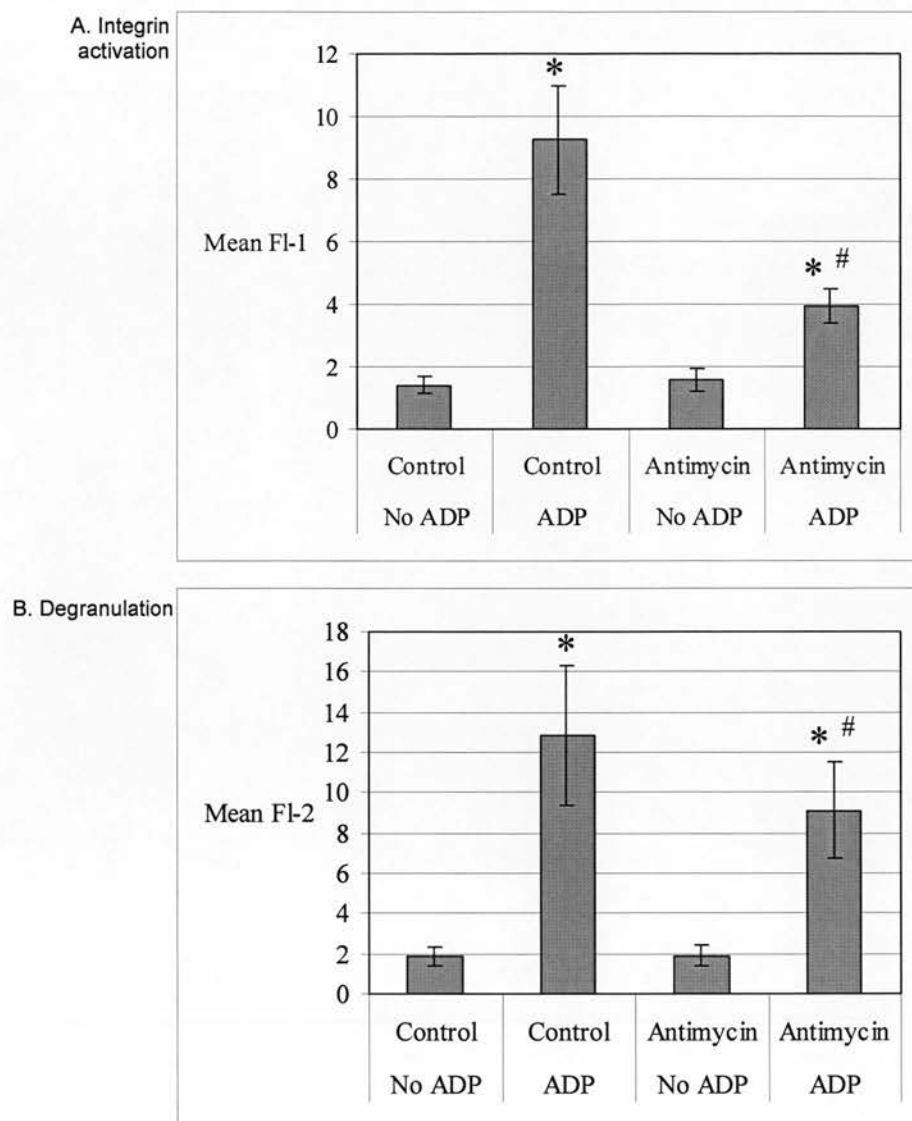


Figure 6.12. Activation of $\alpha_{IIb}\beta_3$ and expression of CD62P in response to ADP is reduced by Antimycin A.

To confirm that Antimycin A had bioactivity platelets (as PRP) were incubated at 37°C for 1 hour in the presence of either 10µg/ml Antimycin A or vehicle control (EtOH to a final concentration of 0.1%) prior to activation in the presence of 2mM CaCl_2 with 5µM ADP. The binding of Alexa⁴⁸⁸-conjugated fibrinogen to platelets was used to assess integrin activation (A) and CD62P expression was assessed with phycoerythrin-conjugated anti-CD62P antibodies (B). $n = 4$; * $P < 0.05$ from non activated control; # $P < 0.05$ between activated in the presence or absence of Antimycin.

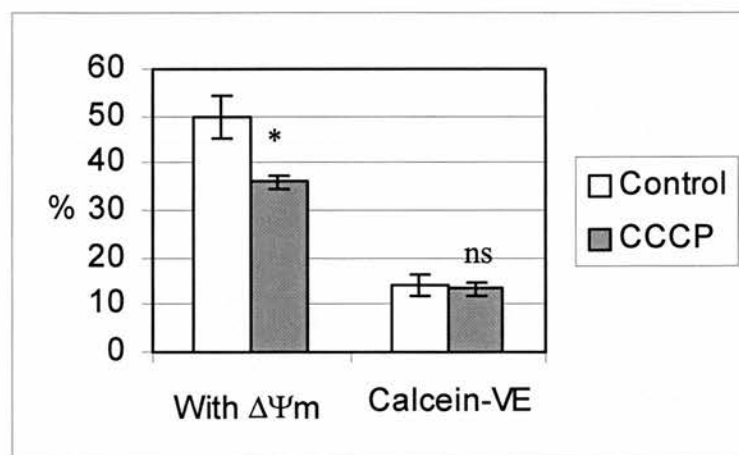


Figure 6.13. Loss of $\Delta\Psi_m$ is uncoupled from loss of platelet viability. Platelets were aged for 24 hours in the absence or presence of 10 μ M CCCP and examined for viability using calcein and for loss of $\Delta\Psi_m$ using JC-1. Although CCCP negatively affected $\Delta\Psi_m$ this did not lead to a change in the frequency of dead platelet by 24 hours. N=3; *P<0.05. ns = no significant difference between treatments.

it is transported into cells, phosphorylated and accumulates as 2DG-6-phosphate which is not metabolised further, therefore glycolytic flux is inhibited) in the presence of antimycin caused complete loss of their ability to accumulate NAO and a promotion of death as assessed by calcein-AM accumulation (21.5 \pm 2.8% NAO negative for control platelets at 24 hours; 44.1 \pm 5.0%, 22.1 \pm 3.7% and 98.0 \pm 0.1% NAO negative platelets incubated with antimycin) (fig. 6.14).

6.2.9 BH3I-2' combines with hypoglycaemia to bring about platelet death.

That antimycin was unable to affect platelet viability on its own but that it showed synergy with hypoglycaemia appeared, at first, to contradict the lack of importance that mitochondrial function has regarding the maintenance of platelet viability *in vitro*. However, it has been reported that, apart from antimycin's known effects upon electron transport, the agent can interfere with pro and anti-apoptotic Bcl-2 family member interactions and lead to the promotion of apoptosis in a murine hepatocyte cell line overexpressing Bcl-X_L (Tzung *et al.*, 2001). Fortuitously, the research article regarding the pro-apoptotic effects of antimycin was published back-to-back with an article regarding small molecule inhibitors of the BH3 domain interaction between pro and anti-apoptotic Bcl-2 family members (Degterev *et al.*, 2001). The most potent molecule described, BH3I-2', was therefore used in conjunction with hypoglycaemia in order to see if it could recapitulate the effects of antimycin.

Incubation of platelets at 37°C for 24 hours with BH3I-2' (20 μ M) did not result in increased platelet death according to the calcein accumulation assay (13.3 \pm 1.0%

control; 12.7 \pm 1.1% with BH3I-2'; $n = 3$; $P=0.63$), however if platelets were maintained under both hypoglycaemic conditions and with BH3I-2' there was substantial death within 24 hours (13.3 \pm 2.0% control; 69.3 \pm 11.0% with BH3I-2'; $n = 3$ different donors; $P<0.01$) (fig. 6.15). Thus, BH3I-2' recapitulated the results obtained with antimycin.

6.2.10 BH3I-2' dramatically affects the fluorescence emission spectra of both JC-1 and NAO.

Platelets treated with BH3I-2' always displayed dramatic but unusual changes in their ability to accumulate JC-1 or NAO. Platelets treated with BH3I-2' and then stained with JC-1 and examined by cytometry showed a marked shift in both red and green fluorescence, whereas BH3I-2' treated platelets stained with NAO failed to accumulate the dye to the same fluorescence intensity as controls. These data initially were thought to mean that platelet mitochondrial biology had become modified by BH3I-2' but it was unclear why JC-1 fluorescence failed to describe loss of $\Delta\Psi_m$ in the normal manner (loss of red fluorescence and simultaneous gain in green fluorescence). Thus it was hypothesised that BH3I-2' was directly interfering with fluorescence properties of both NAO and JC-1.

To explore this possibility JC-1 or NAO were mixed with BH3I-2' at the concentrations used within the assays using platelet free plasma diluted 1:500 with calcium free HBSS as diluent. The solutions were scanned for emission spectra at the excitation wavelength used for cytometric analysis (488nm). It was found that the emission spectra of both

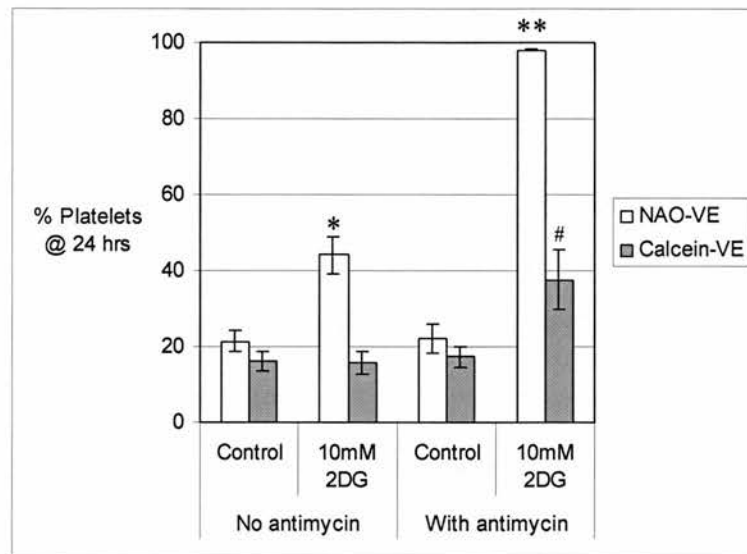


Figure 6.14. Hypoglycaemia and antimycin synergise to bring about platelet death.

Platelets were incubated for 24 hours as cPRP at 37°C with or without 10mM 2DG in the presence or absence of 2µg/ml antimycin. Platelets were then stained with 100ng/ml NAO or 100nM calcein-AM. A discrepancy arises in the abilities of calcein AND NAO to report upon platelet viability under hypoglycaemic conditions. Neither hypoglycaemic conditions nor the presence of antimycin affected platelet viability according to calcein-AM accumulation assay relative to controls. Antimycin with hypoglycaemia acts synergistically to promote loss of platelet viability. $n = 3$; * $P < 0.05$; +/-SE.

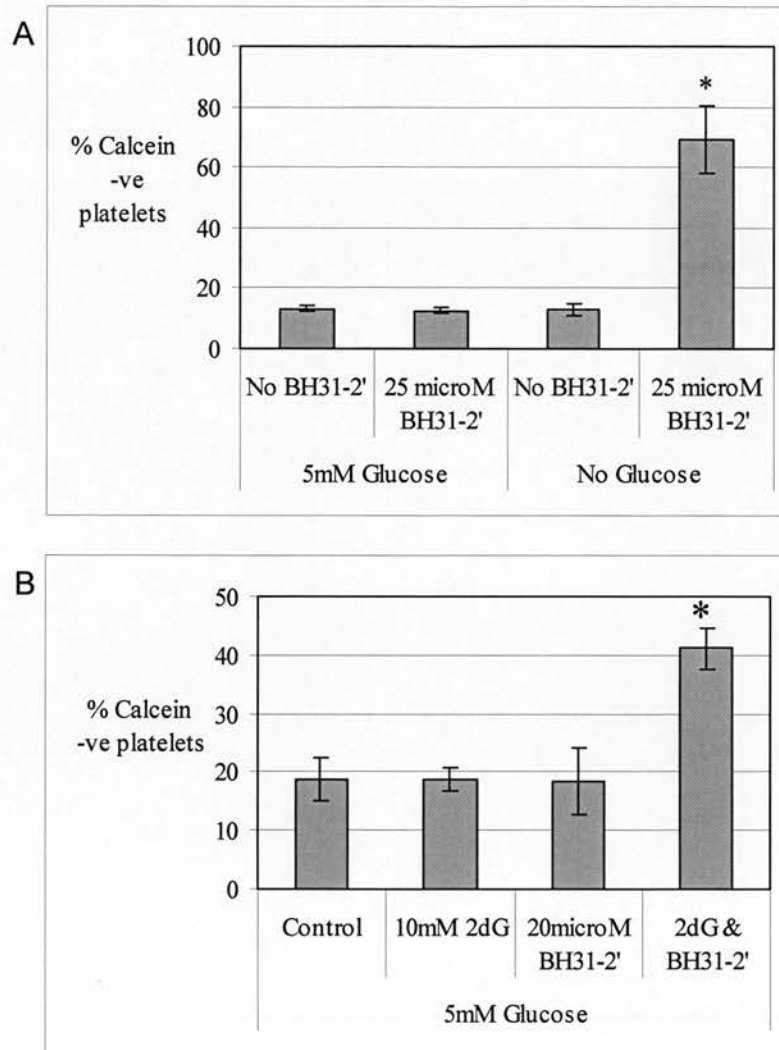


Figure 6.15. Hypoglycaemia and the pro-apoptotic molecule BH31-2' synergise to bring about platelet death.

Platelets were incubated for 24 hours as a 50-fold dilution of PRP with or without glucose to 5mM in the presence or absence of the pro-apoptotic molecule BH31-2' (A). Viability was assessed at 24 hours using calcein accumulation as a marker of viability. Neither hypoglycaemic conditions nor the presence of BH31-2' affected platelet viability compared to controls, however act synergistically to promote loss of platelet viability. A similar experiment with platelets in full PRP and using 2DG to induce hypoglycaemia recapitulated the results using diluted PRP (B) $n = 3$; * $P < 0.05$; \pm SE.

NAO and JC-1 were significantly changed when in the presence of BH3I-2' (fig. 6.16), confirming that the agent directly interfered with their fluorescence characteristics and that the data obtained with these dye combinations could not be relied upon.

6.2.11 Hypoglycaemia leads to changes in platelet calcium_(i).

To understand why hypoglycaemia made platelets more susceptible to agents that are capable of shifting cells towards a pro-apoptotic state the intraplatelet calcium concentration in fresh platelets and platelets incubated for 24 hours at 37°C was examined using Fluo-3 (fig. 6.17). The rationale for looking at calcium_(i) was based on the many links between calcium_(i), mitochondrial dysfunction and cell death (Lemasters et al., 1998; Ermak and Davies, 2002). Strikingly, it was noted that the calcium_(i) of platelets did not increase significantly during 24 hours *in vitro* at 37°C (5.5 +/-0.8mfu and 6.4 +/-0.9mfu for fresh and incubated platelets, respectively; $n = 3$; $P=0.60$). To confirm that Fluo-3 was capable of detecting changes to platelet calcium_(i) freshly isolated platelets were activated with convulxin. CVX-activated platelets exhibited a 6-fold increase in Fluo-3 fluorescence compared to unactivated controls (5.5 +/-0.8mfu and 31.0 +/-0.5mfu for unstimulated and activated platelets respectively; $P<0.001$; $n > 3$), indicating that rises in calcium_(i) are readily detected by this method.

In contrast to this result platelets held under hypoglycaemic conditions (invoked by the presence of 10mM 2DG) exhibited a 2-fold increase in Fluo-3 fluorescence (from 6.4

+/-0.9mfu to 11.8 +/-1.6mfu; $n = 4$; $P < 0.05$) (fig. 6.18), indicative of a change in the platelets' ability to regulate calcium homeostasis under hypoglycaemic conditions.

Citrated plasma retains a free calcium concentration of approximately 50 to 60 μ M, thus it is possible for increases in intraplatelet calcium to be due both to an *efflux* from stores and an *influx* from the plasma. Influx of calcium due to capacitative calcium entry via store operated calcium channels is known to be inhibited by 2-aminoethoxydiphenyl borate (2-APB) (Dobrydneva and Blackmore, 2001), however this drug is known also to inhibit IP₃-sensitive calcium channels (Missiaen *et al.*, 2001). In view of this it was reasoned that incubation of platelets under hypoglycaemic conditions with wortmannin (to inhibit IP₃-sensitive calcium channels) and / or 2-APB would resolve the origin of any intraplatelet calcium rise.

The rise of calcium_(i) caused by hypoglycaemia could be inhibited by 2-APB but not by wortmannin (11.8 +/-1.6mfu; 5.6 +/-0.8mfu and 11.6 +/-1.7mfu for 2DG, 2DG with 2-APB and 2DG with wortmannin, respectively), indicating that IP₃-sensitive calcium channels were not being activated either in controls or by hypoglycaemia. This result is consistent with the hypothesis that extracellular glucose mediates calcium homeostasis in human platelets. However, inhibition by 2-APB of the increase in calcium_(i) that was caused by hypoglycaemia had no effect on the frequency of platelets' able to accumulate NAO, indicating that the influx of calcium was not responsible for the increased number of platelets unable to accumulate NAO. The lack of increased calcium in controls suggests that platelet death *in vitro* is not the result of calcium_(ex) influx or calcium efflux from internal stores. The effect of 2-APB was to return calcium_(i) levels

to controls, suggesting that a calcium efflux from stores did not contribute to the increased calcium_(i).

6.2.12 Hexokinase I does not appear to accumulate in the cytoplasm as a result of hypoglycaemia.

It was hypothesised that mitochondrial integrity had been affected by hypoglycaemic conditions. It has been reported that 2DG-induced hypoglycaemia can cause production of reactive oxygen species and oxidation of cardiolipin (Nomura et al., 1999; Nomura et al., 2000). Furthermore, due to the recent work demonstrating a role for hexokinase in the regulation of apoptosis, oxidation of cardiolipin may have been associated with the dissociation of hexokinase from the mitochondrial outer membrane (Pastorino et al., 2002; Danial et al., 2003).

Western blot analysis of platelet proteins obtained after sonication and isolation of cytosolic and membrane-enriched fractions using antibodies to human Hexokinase I revealed the presence of two major immuno-reactive polypeptides of approximately 100 and 50kDa (fig. 6.19). However there was no qualitative difference in the signal intensities from platelets incubated overnight under hypoglycaemic conditions compared to controls incubated with 5mM glucose.

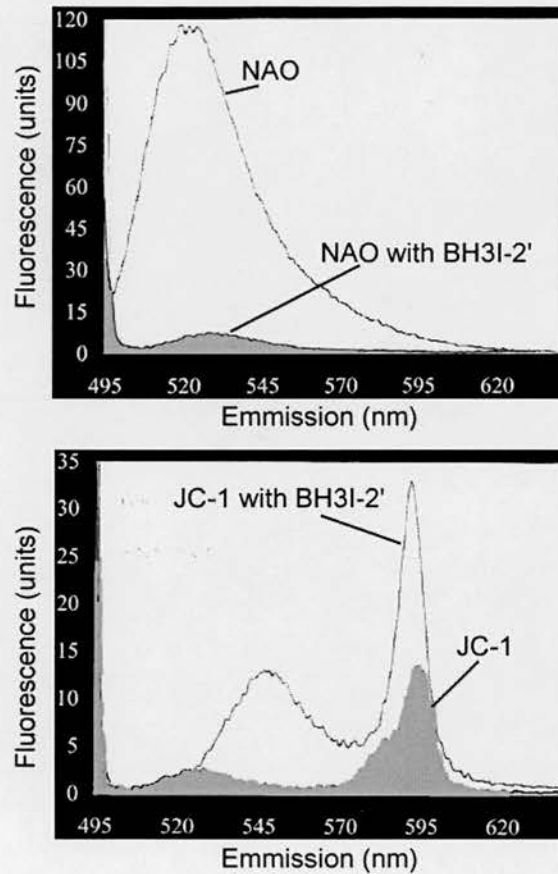


Figure 6.16. BH3I-2' affects the emission spectra of NAO and JC-1.

The emission spectra of NAO and JC-1 were analysed in platelet-free solution (cPPP diluted 1:500 with HBSS). The emission spectra were then re-assessed in the presence of BH3I-2'. The concentrations used were the same as used for the assessment of platelet NAO accumulation or $\Delta\Psi_m$ by JC-1. A clear quantitative reduction in the fluorescence intensity of NAO is caused by BH3I-2'. Furthermore, BH3I-2' alters the intensity and emission spectrum JC-1.

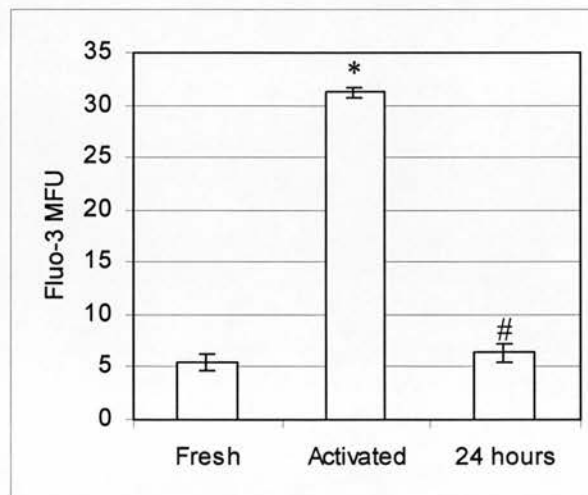
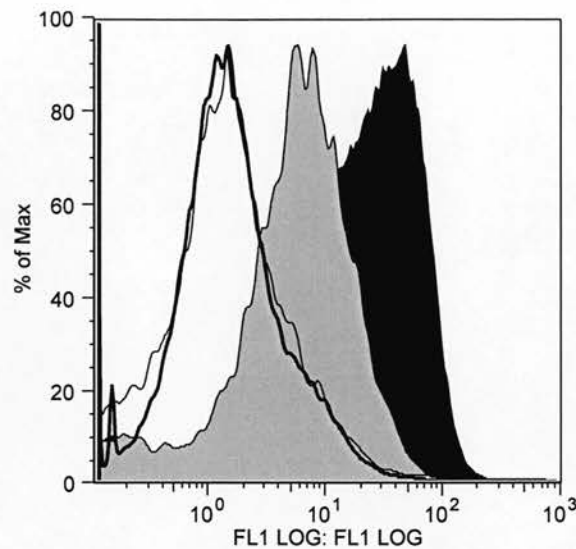


Figure 6.17. Changes in intraplatelet calcium during *in vitro* incubation and in response to various treatments.

Platelets were loaded with Fluo-3 for 30 minutes at 37°C when fresh or after 24 hours *in vitro*. Fresh platelets and platelets aged for 24 hours exhibited similar Fluo-3 fluorescence signals (light and heavy lines in the histogram) whereas platelets incubated with 2-deoxy D-Glucose (2DG) had increased Fluo-3 fluorescence signal (grey peak in histogram). As a positive control fresh platelets were activated with convulxin, which led to a 2-log order shift in Fluo-3 fluorescence (black peak in histogram). Quantified data (bar chart) from 3 donors indicated that although calcium signal could be detected after activation with convulxin there was no increased cytoplasmic calcium detected by Fluo-3 when platelets had been incubated for 24 hours. n = 3; +/-SE; *P<0.01; #Not significantly different from fresh.

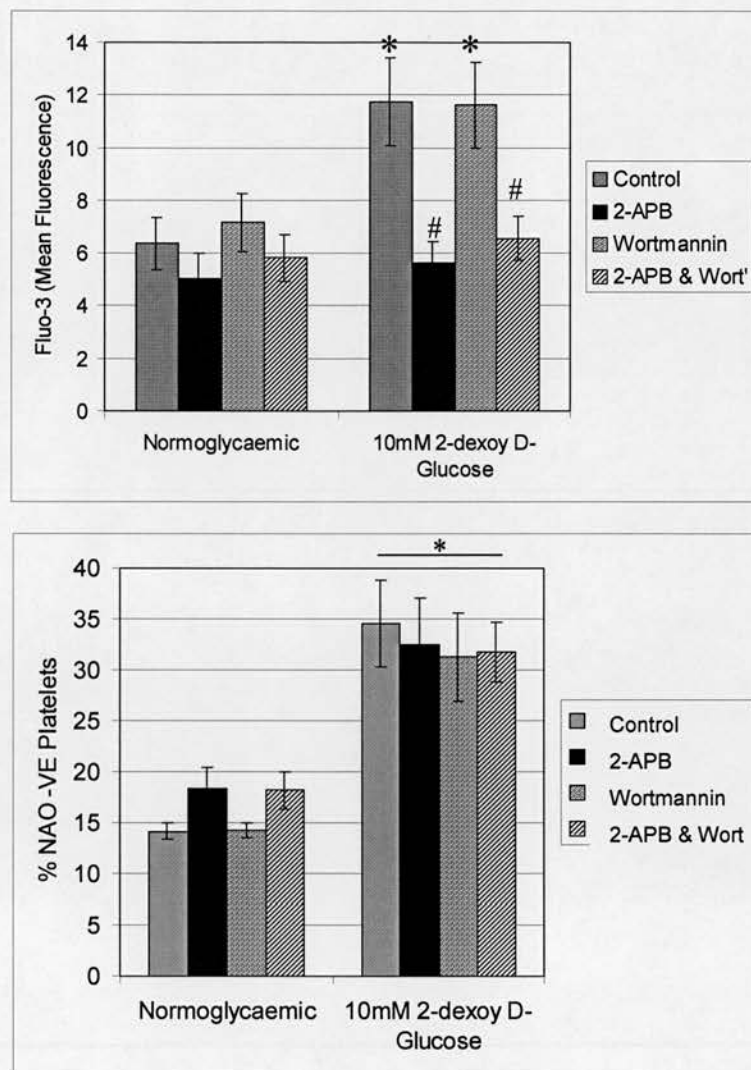


Figure 6.18. Effect hypoglycaemia, 2-APB and wortmannin on the ability of platelets to accumulate nonyl acridin orange.

Platelets were aged for 24 hours in the presence or absence of 2DG and various pharmacological agents. Platelets were then assessed for cytoplasmic calcium and their ability to accumulate 10-*N*-nonyl acridine orange by flow cytometry. The increased calcium signal caused by 2DG was inhibited by 2-APB but not wortmannin however none of these treatments had any effect on the platelets inability to accumulate NAO. $n = 4$; \pm SE; * $P < 0.01$ from normoglycaemic controls; # $P < 0.01$ from 2DG treated group & not significantly different from normoglycaemic controls.

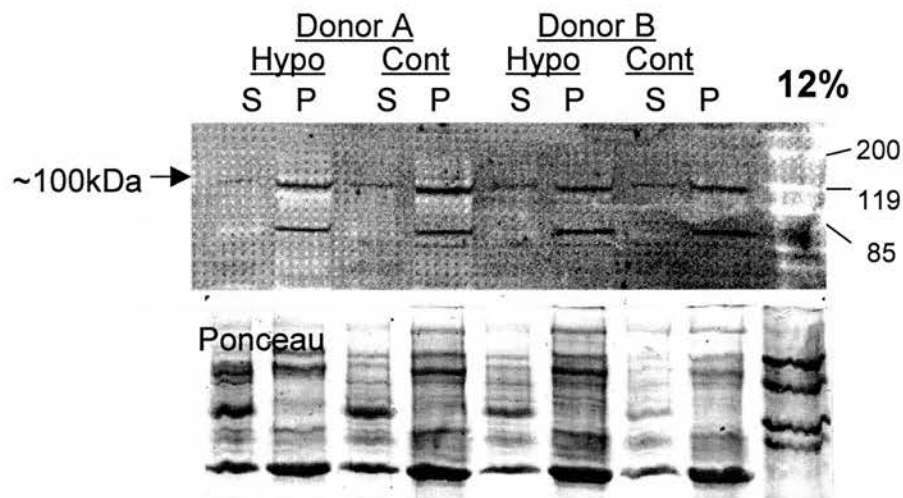


Figure 6.19. Changes to sub cellular localisation of Hexokinase I are not discernible in response to 2DG.

Platelets were aged for 24 hours in the presence or absence of 2DG, lysed by hypotonic shock and sonication, centrifuged to form membrane rich and cytosolic fractions and examined by western blotting using antibodies to Hexokinase I. Hexokinase is an approximately 100kDa protein, two immuno-reactive bands can be seen one at ~100kDa and another at 70kDa. Ponceau stained blot confirmed equal loading of protein into gels. Hypo = hypoglycaemic samples; Cont = normoglycaemic samples; S = (cytosolic fraction; P = membrane fraction)

6.3 Discussion.

Platelets surface express GLUT3 receptors when stimulated by thrombin (Sorbara *et al.*, 1997) and are sensitive to inhibitors of electron transport such as antimycin during activation (McElroy *et al.*, 1971; Peerschke, 1999). However, in their resting state most energy is provided by anaerobic glycolysis (Sorbara *et al.*, 1997). It has been known for many decades that platelets possess glycogen (Zingoni, 1952) and that glycogen becomes depleted during activation (Scott, 1967) due to the direct positive effects of increased calcium_(i) upon glycogen phosphorylase activity (Gear and Schneider, 1975). It was therefore hypothesised that platelet viability is maintained by catabolism of glycogen during *in vitro* storage.

It was demonstrated that platelets possess large PAS-positive inclusions at the light microscopic level and that at the TEM level fields of glycogen are present. It was shown using the anthrone assay that the glycogen content of platelets became reduced during *in vitro* storage at 37°C, a finding that is in agreement with other studies (Zingoni, 1952; Scott, 1967; White *et al.*, 1980). Glycogen was lost from platelets just as rapidly in the absence of glucose_(ex), which is consistent with the hypothesis that the glucose requirements of platelets are met primarily by the catabolism of glycogen.

The importance of glycogenolysis to platelet viability was examined using three distinct pharmacological agents; caffeine, lithium and CP-91149, which have broad range of pharmacological targets but overlap occurs at the point of glycogen breakdown (Martin et al., 1998; Zhang et al., 2003). Lithium is able to promote glycogen synthesis by blocking the dephosphorylation of GSK3 and it is thought that glycogen synthase and phosphorylase activities exists in a 'see-saw' state that when one is activated the other is inactivated, thus facilitating the rapid anabolism or catabolism of glycogen as required by the body (Latsis et al., 2002; Aiston et al., 2003). Although structurally similar to caffeine it is believed that CP-91149 acts upon glycogen phosphorylase by maintaining the enzyme in an inactive dephosphorylated state (Latsis *et al.*, 2002) (see schematic in fig. 6.20). As expected, all three agents were capable of inhibiting glycogen breakdown in platelets stored at 37°C for 24 hours, however all were also toxic to platelets, forging a link between the liberation of glucose from glycogen and the maintenance of platelet viability. Although it is entirely reasonable that the toxicity associated with any of these agents was the cause rather than the effect of inhibited glycogenolysis it is felt that the use of three distinct inhibitors supports the hypothesis that platelet viability *in vitro* is maintained by an ability to liberate glucose from glycogen.

Whether or not the loss of glycogen from platelets stored *in vitro*, which many groups have observed, is an artefact of the *in vitro* environment is not entirely clear. However, one report regarding platelets held for 72 hours at 22°C reported morphological changes similar to those reported in this thesis (loss of the ability to aggregate; 'disc to sphere

transformation' and loss of glycogen). The authors observed that platelets stored for 24 hours and transfused into a thrombocytopenic patient appeared to regain not only their ability to aggregate but also their glycogen, which increased to control levels within 24 hours post transfusion (Murphy and Gardner, 1971). This ability of stored platelets to 'recuperate' upon transfusion has also been observed by other groups (Vainer et al., 1976; Rinder et al., 2003) and lends weight to the possibility that the lesion associated with platelet storage does not reflect the platelets' natural development of senescence but is rather an artefact of their *in vitro* environment. It is important to note that calcium_(i) was not significantly increased in platelets incubated for 24 hours at 37°C, thus it appears unlikely that phosphorylase activity and glycogen catabolism was being regulated by calcium. In other cells phosphorylase is regulated by PKA, but PKA activity in platelets could not be implicated in the breakdown of glycogen (see appendix figure 8.6). Therefore it is possible that the dephosphorylation of glycogen phosphorylase (by phosphoprotein phosphatase-1 (PP-1)) is not occurring *in vitro* and that phosphorylase activity is constitutively 'switched on'. Examination of the phosphorylation states of glycogen phosphorylase and the PP-1 is required to explore this possibility.

A major confounding factor in this research programme is the possible effect of citrate upon glycolytic flux. Citrate is an allosteric inhibitor of phosphofructokinase, thus anticoagulation of whole blood using citrate is theoretically able to inhibit glycolysis, however reports of citrate usage by platelets are contradictory and not easily compared due to being performed at different temperatures with platelets from different species.

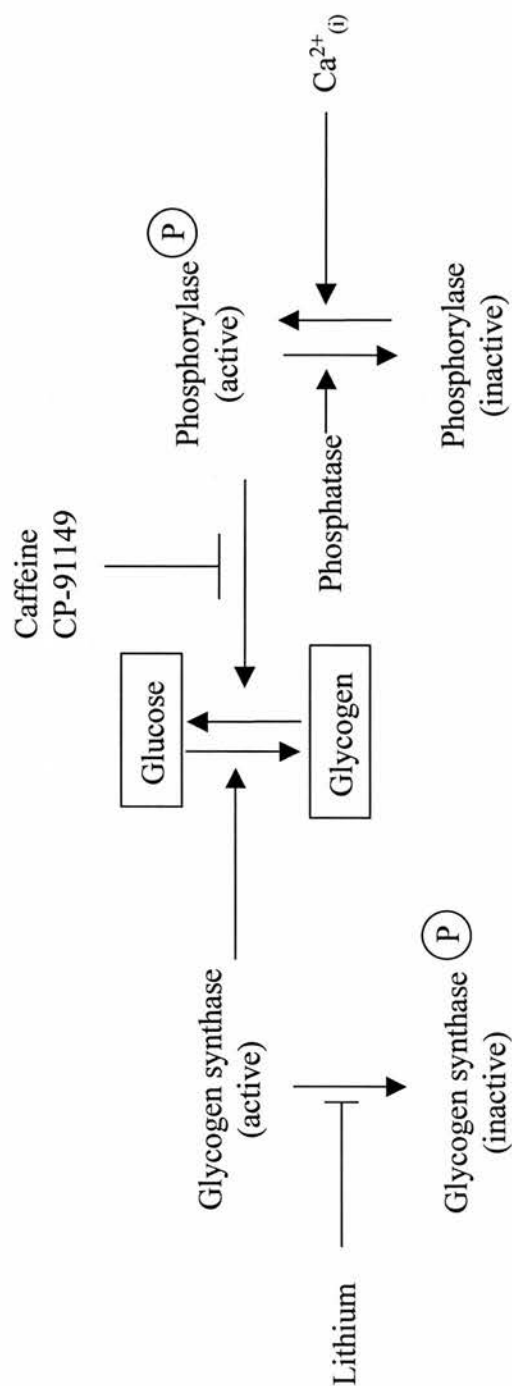


Figure 6.20. Highly simplified schematic representing effects of caffeine, lithium and CP-91149 on glycogen metabolism.
 Glycogen catabolism in activated platelets is promoted by effects of increase in calcium(i) upon phosphorylase activity. Phosphorylase is inhibited directly by caffeine which acts at the catalytic site and by CP-91149 which prevents phosphorylation of the enzyme. Lithium promotes glycogen synthesis by inhibiting the dephosphorylation of GSK3 and maintaining it in an inactive state, that is unable to phosphorylate and inactivate glycogen synthase.

Some authors indicate that platelets are unable to utilise exogenous citrate (Tegos and Beutler, 1979), whereas others suggest that platelets can (Detwiler and Zivkovic, 1970), or do so at an albeit very slow rate (Cartledge *et al.*, 1997). Considering the fact that platelets washed from citrated plasma died at a similar rate as those in full citrated plasma or diluted plasma (see chapter 3), the effect of citrate in the current research program upon platelet death may be negligible. Further examination of this possibility by anticoagulating blood with EDTA and repetition of key experiments is required to establish if glycolytic flux is normal in cPRP.

The incubation of platelets under hypoglycaemic conditions highlighted a discrepancy between the calcein and NAO accumulation assays. Consistently within this research program the NAO and calcein-AM accumulation assays appeared to report the same event - loss of viability in platelets; however hypoglycaemic conditions increased the frequency of NAO^{-VE} platelets but had no effect upon the frequency of calcein^{-VE} platelets. NAO is specific for the mitochondrial lipid cardiolipin but has been reported to extrude from depolarising mitochondria, suggesting that it is capable of reporting changes to $\Delta\Psi_m$ (Jacobson *et al.*, 2002). It is worth noting that changes to cardiolipin and $\Delta\Psi_m$ have not been shown to occur exclusively of each other in the context of cell death. The discrepancy suggested that platelets were sensitive to altered environmental glucose concentrations but that they had little influence upon platelet viability (even over the extended period of five days). The discrepancy highlighted also the possibility that changes to mitochondrial $\Delta\Psi_m$ have no direct effect upon the viability of platelets *in vitro*. Several further lines of evidence support this conclusion; it was demonstrated

that over a five-day period of incubation at 37°C the rate death and loss of $\Delta\Psi_m$ were dissimilar from each other, linear and hyperbolic, respectively; mCCCCP failed to promote platelet death and, finally, antimycin failed also to promote platelet death. This raises the possibility that platelets contain 'get out clause' that allows them to avoid a mitochondrial driven form of cell death. It also brings into question the use of prononophores by some research groups (Bergmeier et al., 2003) to recapitulate platelet storage lesion *in vitro* (especially when it occurs spontaneously).

The hypothetical 'get out clause' protecting platelets from changes to $\Delta\Psi_m$ and / or cardiolipin is possibly the target of antimycin and BH3I-2'. These agents in their own right had no significant effect on platelet viability, yet there was considerable synergy between antimycin or BH3I-2' and hypoglycaemia. It has been demonstrated that antimycin (Tzung *et al.*, 2001) and BH3I-2' (Degterev *et al.*, 2001) share an ability to bind the hydrophobic groove of Bcl-2 and Bcl-X_L, thus inhibiting their interaction with pro-apoptotic Bcl-2 family member proteins (such as Bax, Bak and Bik). The consequence of this (at least for BH3I-2'-treated cells) is the promotion of mitochondrial swelling, loss of $\Delta\Psi_m$ and cell death by apoptosis in a Bax-dependent manner (Feng *et al.*, 2003). Thus, treatment of platelets with these agents may increase the concentration of free pro-apoptotic Bcl-2 family members in platelets, but that this increase is not sufficient to promote platelet death. Examination of Bcl-X_L binding partners by immunoprecipitation of platelet lysates using anti-Bcl-X_L antibodies may help resolve whether antimycin or BH3I-2' affect the heterodimerisation of this molecule with pro-apoptotic members of the Bcl-2 family.

If an increase in the pro-apoptotic balance within platelets is not sufficient to cause death what does hypoglycaemia do that facilitates the toxic effects of BH3I-2' or antimycin?

Evidence of a calcium leak from internal store(s) was provided by the ability of 2-APB but not wortmannin to block the increase in calcium_(i) caused by hypoglycaemia. Although the changes to NAO accumulation caused by hypoglycaemia were not dependent upon this increased calcium_(i) it is a fascinating observation that low environmental glucose appears to be a regulator of calcium homeostasis in platelets. The observation that 2-APB was able to completely block the rise in calcium_(i) suggests rapid sequestration of store-leaked calcium was occurring. It is known that certain organelles utilise locally produced ATP for pumping calcium (e.g. the endoplasmic reticulum (Xu *et al.*, 1995)) and it is becomingly increasingly clear that mitochondria have 'privileged' access to calcium released from the endoplasmic reticulum (Srivastava *et al.*, 1999) and that stress of the ER leading to calcium release can promote loss of $\Delta\Psi_m$ and subsequent cell death (Chae *et al.*, 2004). However, it is known also that cell death effected by hypoglycaemic stress can be mediated by dissociation of hexokinase IV (glucokinase) from mitochondria and promotion of apoptotic cell death (Danial *et al.*, 2003). The mechanism for hypoglycaemia-induced cell death appears to involve hexokinase dissociation leading to translocation of Bax to the outer mitochondrial membrane and release of cytochrome C (Pastorino *et al.*, 2002).

Furthermore, hexokinase II dissociates from mitochondria in glucose-deprived rat fibroblasts, an event that permits tBid-dependent apoptosis (Majewski *et al.*, 2004).

Taking these observations into account it is hypothesised that platelets rely upon exogenous glucose to maintain hexokinase at the mitochondrial outer membrane and support calcium pumping by the DTS. Low glucose_(ex) promotes a net calcium leak from the DTS which is buffered by mitochondria. The calcium leak initiates store operated calcium channel opening. The calcium buffering by mitochondria affects cardiolipin / $\Delta\Psi_m$ and prevents platelets accumulating NAO. These changes do not lead to platelet death. An increase in the pro-apoptotic balance within the platelets (e.g. by antimycin treatment or BH3I-2') leads to promotion of Bax insertion into the mitochondrial outer membrane due to the loss of hexokinase and subsequent mitochondrial rupture and platelet death (see figure 6.21).

Examination of hexokinase localisation in platelets treated with 10mM 2DG did not reveal an accumulation within the cytosol, however repetition of this experiment is required with confirmation that cytosolic and membrane fractions were suitably pure to ascertain a difference (e.g. by confirming a pure mitochondrial signal in the membrane fraction with antibodies to a subunit of cytochrome oxidase). Furthermore a positive control is required that confirms the accumulation of hexokinase in the cytosol. This could be done by incubating control lysates with G6P in order to inhibit hexokinase

activity and promote it's dissociation from mitochondria and subsequently preparing membrane and cytosolic fractions (Pastorino *et al.*, 2002).

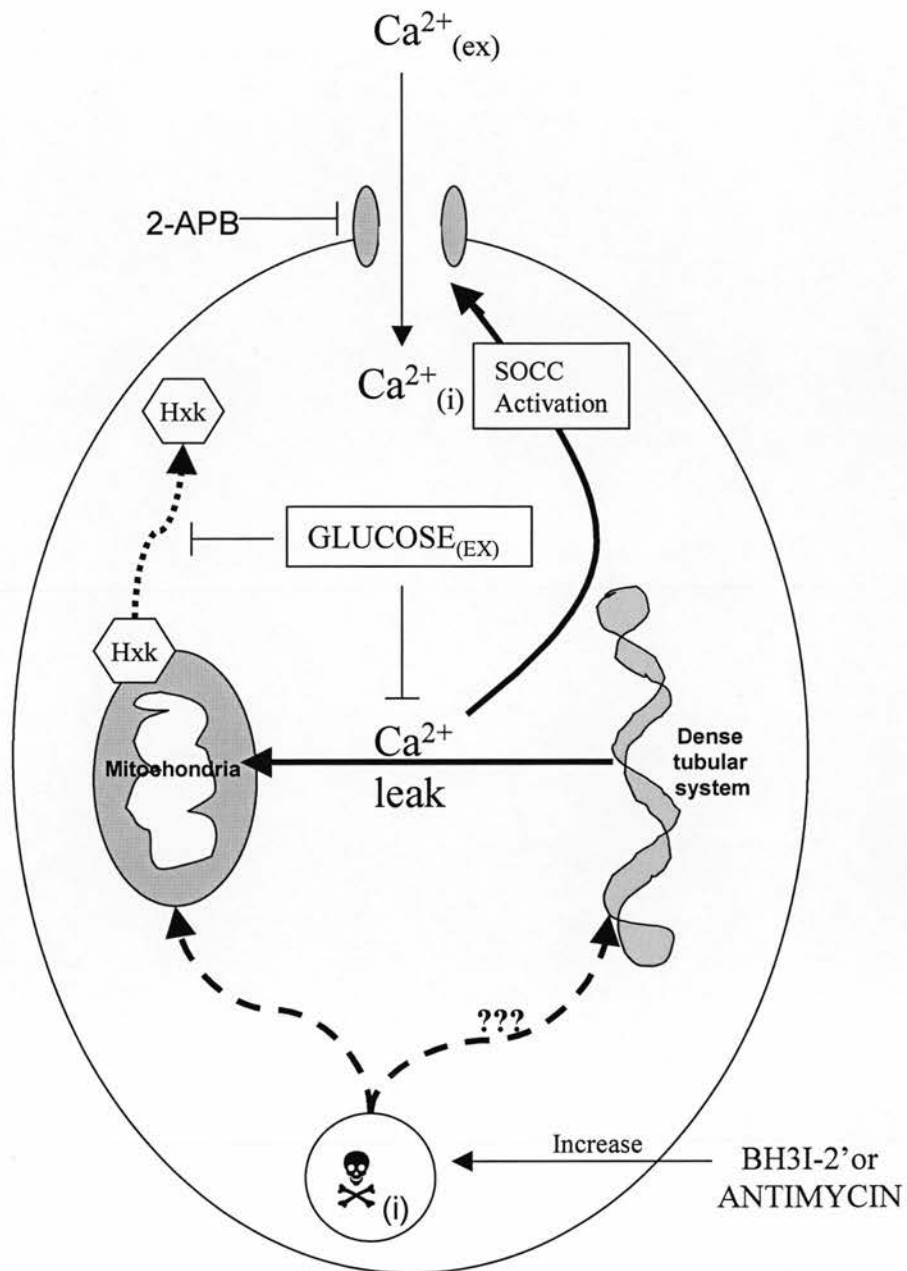


Figure 6.21. Hypothetical model for the toxic effects of BH3I-2' and antimycin.

Platelets incubated for 24 hours under hypoglycaemic conditions display increased calcium_(i). This increase is blocked by 2-APB due to inhibition of store operated calcium channel opening. 2-APB returns calcium_(i) to control levels suggesting that the calcium (leaked from the DTS?) responsible for initiating SOCC opening is fully sequestered (by the mitochondria?). Hexokinase dissociates from the mitochondria due to low glucose_(ex). Increasing the pro-apoptotic balance with antimycin or BH3I-2' allows free Bax to insert into the mitochondrial outer membrane due to the absence of Hexokinase. This allows substantial and rapid mitochondrial disruption and platelet death.

7 CONCLUSIONS

This thesis aimed to readily identify platelet death and understand why platelets die *in vitro*. To perform this work it was necessary to develop a means by which dead platelets could be readily and rapidly discriminated from live ones. The dyes calcein-AM and FM4-64 were found to be useful for this purpose and were used extensively as reporters of platelet viability. It was also found that the dyes could identify platelet microvesiculation, a process that appears to occur within large aggregates.

Once readily identifiable, changes to the platelet surface could be studied. It was found that CD42b, an important component of the vWF receptor complex is lost from platelets at the point of death. The shedding of CD42b was due to metalloproteinase activity. Metalloproteinase activity was also responsible for an interaction between dead platelets. The physiological significance of this interaction is not understood.

Macro-autophagy could not be implicated as the means by which platelets died. It is unlikely that vacuolation represented a form of macroautophagy but it may have been the result of crinophagy. The results did raise the possibility that autophagic pathways may have an important role to play in the maintenance of platelet function.

Glycogen content of platelets became reduced during *in vitro* incubation at 37°C. Inhibitors of glycogen catabolism caused platelet death. Exogenous glucose did not support platelet viability but did appear to maintain calcium homeostasis and mitochondrial integrity as evidenced by an inability to accumulate nonyl acridine orange. Antimycin did not affect the platelets' ability to accumulate NAO and was not toxic to platelets unless in conjunction with hypoglycaemic conditions. Very similar

results were obtained with BH3I-2', suggesting that antimycin and BH3I-2' affect the pro-apoptotic balance within platelets. This pro-death equilibrium is only realised as death -promoting when platelets are held under hypoglycaemic conditions. A great deal more experimental work is required to test this hypothesis but it highlights a need to further understand the role of metabolism in the function of platelets.

8 APPENDIX

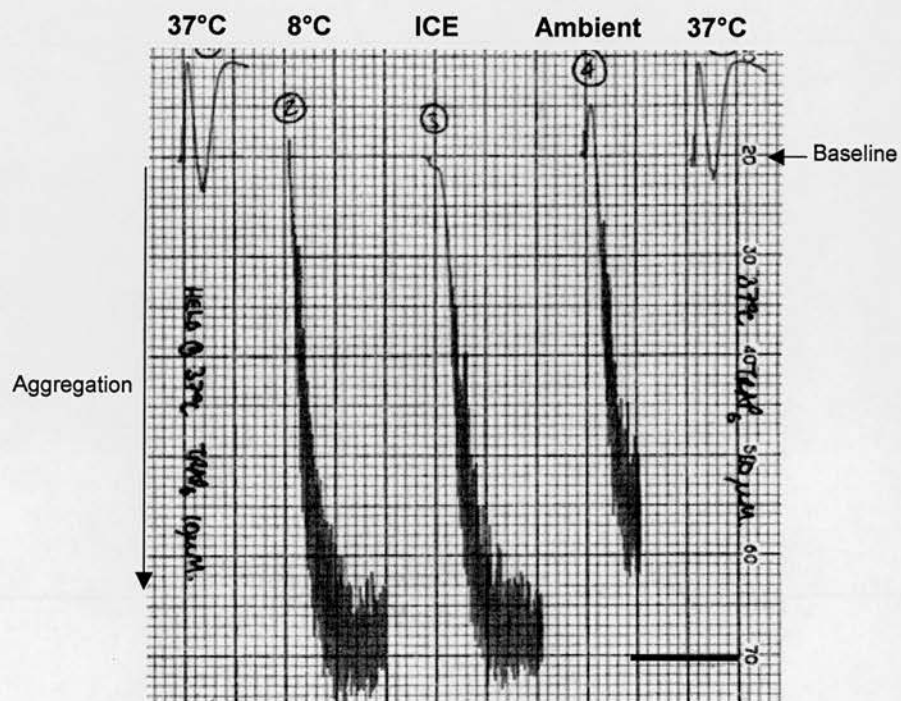


Figure 8.1. Low temperatures prime platelets for activation.

Platelet rich plasma was collected from healthy volunteers and incubated for 10 minutes at the temperatures described in the figure. Platelets were transferred to aggregometry tubes, CaCl_2 and Thrombin receptor activated peptide (TRAP6) was added to 2mM and $10\mu\text{M}$, respectively. Bar = 2 minutes, Maximum aggregation was 80units from baseline.

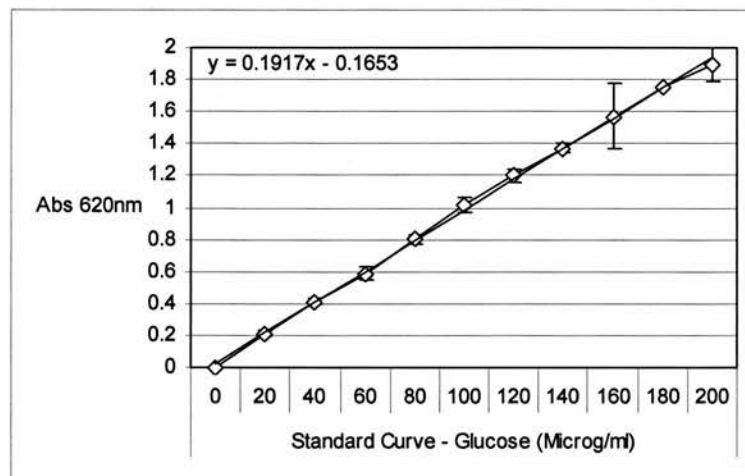


Figure 8.2. Standard curve for Anthrone assay.

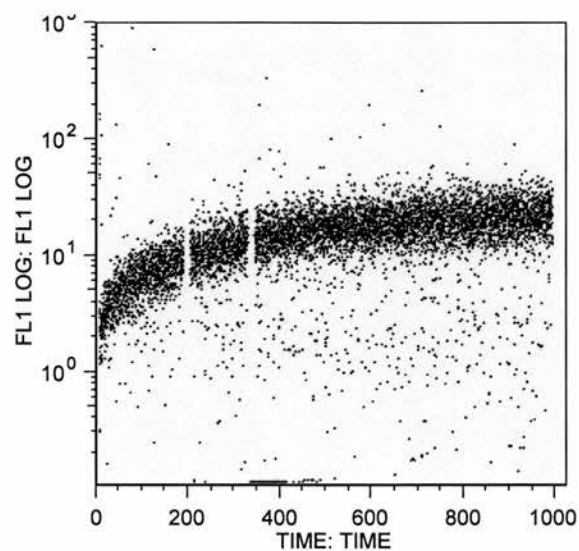


Figure 8.3. Assay for the accumulation of NAO into live platelets.

Platelets were added to a solution of 500pM NAO at and immediately placed into the cytometer. Cytometric analysis of platelet fluorescence was performed for 15 minutes. The accumulation of NAO by platelets was at equilibrium within 10 minutes (600s). This assay system was useful for the determination of accumulation rates for other platelet stains such as calcein-AM and FM4-64.

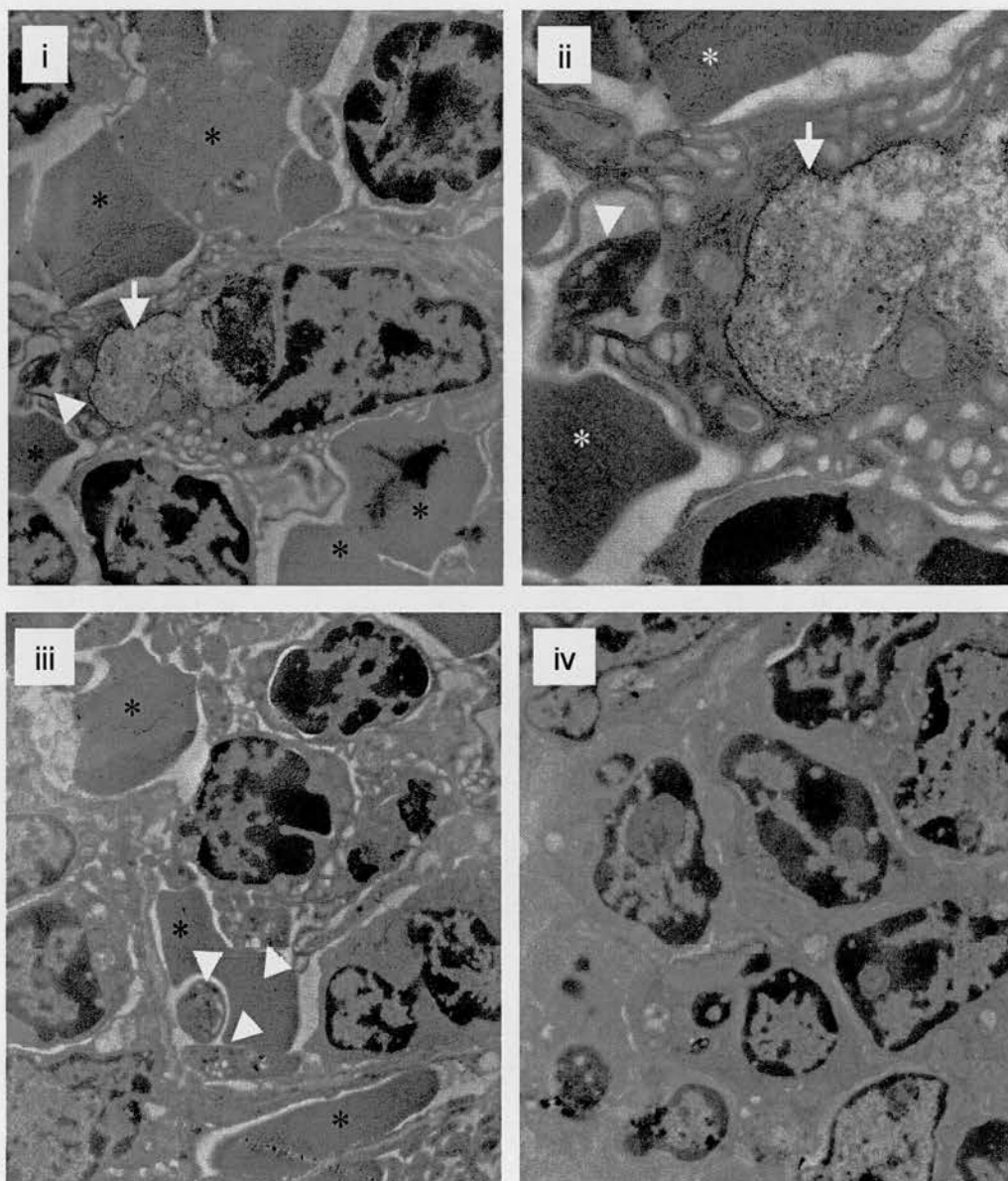


Figure 8.4. Transmission electron micrographs of murine splenic red and white pulp.

Micrographs of platelets within murine splenic red pulp (i to iii) and a micrograph of white pulp for comparison (iv). Platelets (arrowheads) can be seen closely associated with a phagocyte with large phagolysosome (arrow) (i and ii). Other platelets can be seen proximal to erythrocytes (asterisk). All platelets look normal or with signs of activation (i and ii). No platelets could be found with a phenotype similar to that seen during *in vitro* storage at 37°C.

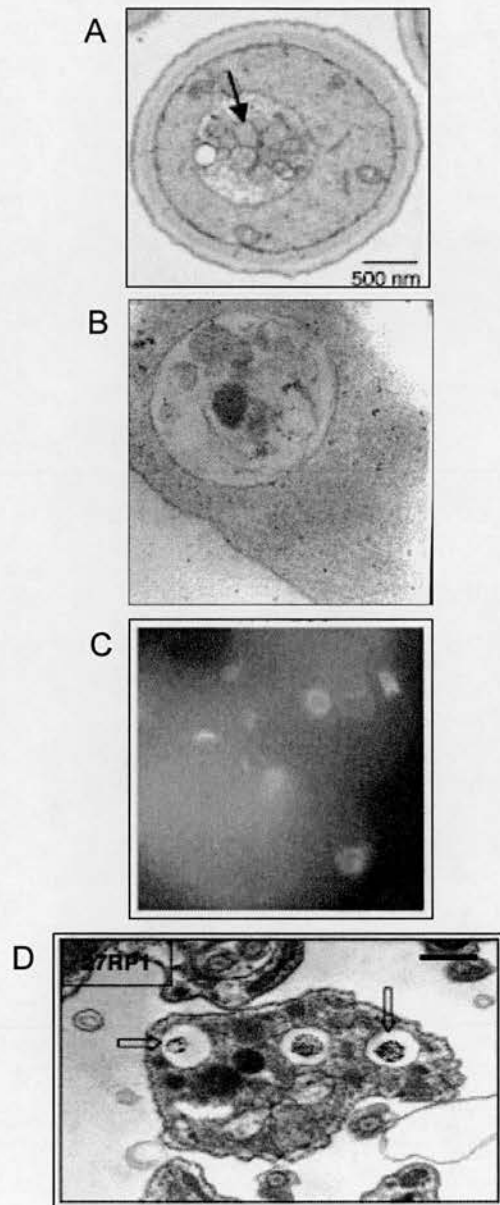


Figure 8.5. Gallery of autophagosomes in various organisms.

(A) Autophagosomes (arrow) in the vacuole in the yeast, *Saccharomyces cerevisiae*, during nitrogen starvation. (B) Granule-filled vacuole in platelets (the present research programme). (C) Yellow-fluorescent protein tagged LC-3 (an autophagosome marker) in ES cells (Mizushima *et al.*, 2003). (D) Autophagic vacuoles in *Leishmania donovani* after antimicrobial peptide treatment (Bera *et al.*, 2003).

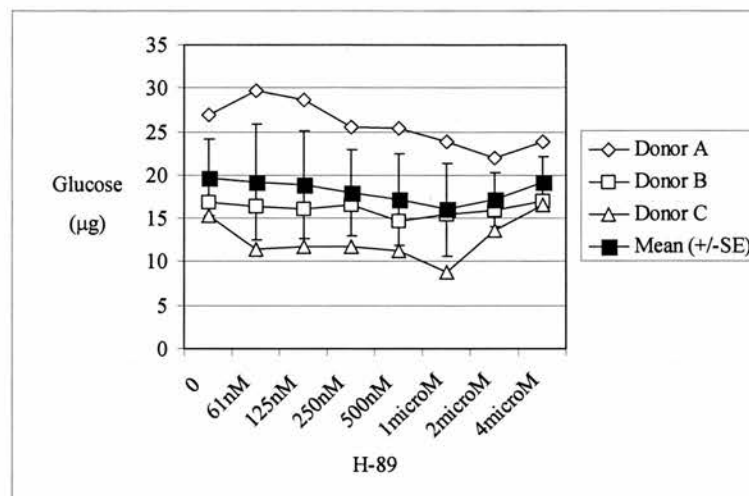


Figure 8.6. Loss of glycogen during *in vitro* storage is independent of Protein Kinase A activity.

Platelets were incubated as cPRP for 24 hours in the presence of the protein kinase A (PKA) inhibitor H-89. A dose-response curve shows a consistent lack of effect on the loss of glycogen from platelets. $n = 3$.

9 REFERENCES.

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